

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Baskaran CHANDRASEKAR		
Docket No.:	3521-101	Confirmation No.	2963
Serial No.:	10/088,405	Group Art:	1617
Filing Date:	July 24, 2002	Examiner:	CARTER, Kendra D.
Title:	LOCAL DELIVERY OF 17-BETA ESTRADIOL FOR PREVENTING VASCULAR INTIMA HYPERPLASIA AND FOR IMPROVING VASCULAR ENDOTHELIUM FUNCTION AFTER VASCULAR INJURY		

**APPELLANT'S BRIEF ON APPEAL PURSUANT TO 37 C.F.R. § 41.37**

Mail stop Appeal Brief - Patents  
Commissioner for Patents  
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U.S.A.

In support of Appellant's Notice of Appeal that was filed in connection with the above-captioned case on March 5, 2008, and with reference to the Final Office action that was mailed in this case on September 6, 2007, submitted herewith is Appellant's Appeal Brief.

**(i) Real party in interest**

The real party in interest in this case is the "INSTITUT DE CARDIOLOGIE DE MONTRÉAL", Montreal, Quebec, Canada, to whom all interest in the present application has been assigned.

**(ii) Related appeals and interferences**

There are no pending appeals or interferences related to this case.

**(iii) Status of claims**

Claims 2, 9, 15 and 19 are cancelled.

Claims 1, 3-8, 10-14, 16-18 and 20-24 are currently pending, stand rejected in the Final Office action mailed September 6, 2007, and are under appeal.

**(iv) Status of amendments**

No amendment has been filed subsequent to the outstanding Final Action.

**(v) Summary of claimed subject matter**

Independent claim 1 is the only independent claim and is directed to a method for improving reendothelization and vascular endothelial function following vascular injury, such as injury associated with percutaneous transluminal coronary angioplasty (PTCA) (for example at page 2, lines 8 to 10 and lines 21 and 22), which comprises administering, at an injured site in the lumen of a blood vessel (i.e. local administration), an effective amount of 17- $\beta$  estradiol or a derivative thereof with a device (e.g., a catheter). The 17- $\beta$  estradiol or a derivative is present in a dose unit of 1 to 5000  $\mu$ g/Kg of patient's body weight. Support for improving reendothelization and vascular endothelial function following vascular injury can be found, for example, at page 21, lines 4 and 5. Support for the administration at an injured site in the lumen of a blood

vessel having suffered vascular injury, can be found, for example, at page 21, lines 3 to 5, and in original claim 1. Support for the 17- $\beta$  estradiol or a derivative being present in a dose unit of 1 to 5000  $\mu\text{g/Kg}$  of patient's body weight can be found, for example, at page 5, lines 23 and 24 and in original claim 4.

**(vi) Grounds of rejection to be reviewed on appeal**

This appeal presents five issues:

1. Whether claim 24 is unpatentable under the second paragraph of 35 U.S.C. § 112.
2. Whether claims 1, 3-4, 8, 10, 12-14, 16-18, 20 and 22-24 are unpatentable under 35 U.S.C. § 103(a), over the combination of U.S. Patent No. 5,866,561 to Mark T. Unga ("Unga"), O'Brien and Bauters *et al.*
3. Whether claims 5-7, 11 and 21 are unpatentable under 35 U.S.C. § 103(a) over the combination of U.S. Patent No. 5,866,561 to Mark T. Unga ("Unga"), O'Brien, Bauters *et al.* and Pitha (US Patent No. 4,727,064).
4. Whether claims 1, 3-4, 8, 10-14, 16-18 and 20-24 are unpatentable under 35 U.S.C. § 103(a) over the combination of U.S. Patent No. 5,866,561 to Mark T. Unga ("Unga"), Fontana (US 5,384,332) and Grainger *et al.* (US 6,117,911).
5. Whether claims 5-7 are unpatentable under 35 U.S.C. § 103(a) over the combination of U.S. Patent No. 5,866,561 to Mark T. Unga ("Unga"), Fontana (US 5,384,332), Grainger *et al.* (US 6,117,911) and Pitha (US Patent No. 4,727,064).

**(vii) Argument**

1. **The rejection of claim 24 under the second paragraph of 35 U.S.C. § 112 is improper and should be reversed.**

Claim 24, which depends from claim 1, is rejected under the second paragraph of 35 U.S.C. § 112 as allegedly being indefinite. The sole basis for this rejection is that the term "therapeutic moiety" in claim 24 lacks antecedent basis in claim 1. Notably, the final Action does not allege that the offending term itself is in any way unclear, or that the scope of claim 24 cannot be ascertained. That alone is fatal to the rejection. Moreover, Appellant respectfully submits that the term "therapeutic moiety" is well known in the art to refer to any chemical entity having a therapeutic effect, and would be clearly understood as such by the skilled person. That the term is not recited *per se* in claim 1 is not relevant. Claim 24 requires that the therapeutic moiety be "administered." The thing administered in claim 1 is "17- $\beta$  estradiol or a derivative thereof" in an effective amount to improve reendothelialization and vascular endothelial function. There is no reasonable argument that "therapeutic moiety" in claim 24 refers to anything other than "17- $\beta$  estradiol or a derivative thereof," and that the latter term by implication provides antecedent basis for the former. *Energizer Holdings, Inc. v. Intn'l Trade Comm'n.*, 435 F.3d 1366, 1370, 77 USPQ2d 1625, 1628 Fed. Cir. 2006) ("When the meaning of the claim would reasonably be understood by persons of ordinary skill when read in light of the specification, the claim is not subject to invalidity upon departure from the protocol of 'antecedent basis'"). Further, as noted in the MPEP, "[o]bviously, however, the failure to provide explicit antecedent basis for terms does not always render a claim indefinite. If the scope of a claim would be reasonably ascertainable by

those skilled in the art, then the claim is not indefinite." MPEP § 2173.05(e); see also cases cited therein. In view of the foregoing, Appellant respectfully submits that the scope of claim 24 would be readily ascertained by one of ordinary skill in the art, and thus that claim 24 is not indefinite and is in compliance with 35 U.S.C. 112, second paragraph.

**2. The rejection of claims 1, 3-4, 8, 10, 12-14, 16-18, 20 and 22-24 under 35 U.S.C. § 103(a), as being allegedly obvious over the combination of U.S. Patent No. 5,866,561 to Mark T. Ungs ("Ungs"), O'Brien and Bauters *et al* is improper and should be reversed.**

Ungs (Evidence Appendix at 0044-47) was published on February 2, 1999, less than one year prior to the effective filing date of the present application (September 21, 1999). Appellant respectfully submits that Ungs can only be used in a rejection under 35 U.S.C. 103(a)/102(e).

The Office relies on Ungs as allegedly suggesting the application of estrogen to stenosed, dilated vessels after PTCA to prevent restenosis. At page 4 of the Final Office action mailed September 6, 2007, it is alleged, in reference to a statement found at column 1, lines 49-51, of Ungs: "Ungs teaches ...that administration of estrogen to the stenosed, dilated region after PTCA has been suggested for the purpose of preventing restenosis...and thus teaches administration to an injured site, i.e. one that has been injured by PTCA".

The statement found at column 1, lines 49-51, of Ungs reads as follows: "Administration of estrogen to the stenosed, dilated region after PTCA has thus been suggested for the purposes of preventing restenosis" [emphasis added]. As exemplified

by the use of "thus" and "suggested" in the above-mentioned statement, Ungs is not referring to his own work, but is clearly interpreting the work of others, more particularly two prior art documents discussed at column 1, lines 40-49, of Ungs (U.S. Patent Nos. 5,516,528 (Hughes *et al.*; Evidence Appendix at 0135-0145) and 5,376,652 (Javitt; Evidence Appendix at 0146-0152)). Therefore, the skilled person reading Ungs would be directly led to these two references.<sup>1</sup>

Hughes *et al.* is concerned with oral, transdermal or implant delivery of compositions comprising a combination of estrogen and phytoestrogen and their use to reduce the risk of coronary heart disease (CHD) and osteoporosis in post-menopausal women. Therefore, Hughes *et al.* is clearly directed to oral, daily administration of estrogen and phytoestrogen to decrease osteoporosis- and CHD-related morbidity and mortality in post-menopausal women, and not to administration of estrogen to the stenosed, dilated region after PTCA for the purposes of preventing restenosis.

Javitt discloses a method for preventing or reducing restenosis comprising administration of 27-hydroxycholesterol or related compound or sterol-27-hydroxylase stimulant (e.g., estrogen). Javitt teaches administration through the use of liquid and solid formulations, and also through the use of parenteral injectables (see column 4, lines 16-18). Thus, Javitt teaches systemic (e.g., oral or intravenous) administration of 27-hydroxycholesterol or related compound or sterol-27-hydroxylase stimulant for preventing or treating restenosis following blood vessel injury. It does not disclose

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<sup>1</sup> Portions of the Hughes and Javitt references were discussed at page 6 of the June 5, 2007 Amendment. For the Board's convenience, copies of the entire references are included in the Evidence Appendix.

administration of estrogen to the stenosed, dilated region (i.e. to an injured site) after PTCA for the purposes of preventing restenosis.

In view of the foregoing, Appellant respectfully submits that Ungs has clearly misinterpreted the teaching of the prior art, and that such a misleading assessment of the prior art cannot be relied upon for the present rejection. Appellant respectfully submits that someone considering the prior art documents referred to by Ungs for all they fairly teach would not be left with the suggestion to administer a specific amount of 17- $\beta$  estradiol with a device at an injured site to improve reendothelization and vascular endothelial function following vascular injury, as presently claimed.

Appellant also respectfully submits that Ungs teach away from the present invention. Ungs teaches that PTCA is not always a successful solution or even a viable treatment option (Col. 1, lines 52' and 53) and treatment to increase perfusion to heart tissue (i.e. angiogenesis) in place of PTCA would be desirable (Col. 1, lines 62 and 63). Therefore, Ungs suggests a solution to replace PTCA, while the aim of the present invention is to improve reendothelization and vascular endothelial function following vascular injury, such as an injury following PTCA (and is thus a complement to PTCA). Ungs thus teaches away from Appellant's solution by suggesting that angiogenesis induction by estrogen could replace PTCA, and discourages research in the very field where Appellant made his invention. That is the antithesis of obviousness. *In re Rosenberger*, 386 F.2d 1015, 1018, 156 USPQ 24, 26 (CCPA 1967).

The Action also relies on O'Brien (Evidence Appendix at 0048-55) as allegedly suggesting that restenosis is reduced by the inhibition of proliferation of smooth muscular cells (SMCs). Appellant respectfully disagrees and submits that O'Brien

merely suggests several potential mechanisms of action for the ability of estrogens to modulate restenosis in postmenopausal women, including effects on lipoprotein levels, vasoconstriction and cellular migration/proliferation. O'Brien clearly admits at page 1116, left column, that the results concerning the effect of estrogen on the proliferation of SMCs have been conflicting, and specifically refers to a study in nonhuman primates, the model most closely related to humans, in which it was concluded that estrogens do not inhibit balloon-induced proliferation of SMCs. In any case it is also reiterated that the present claims recite a method of improving reendothelization and vascular endothelial function rather than a method of inhibiting SMCs proliferation. The Board is respectfully referred to Dr. Stack's Declaration of record (Evidence Appendix at 0001-43). This Declaration was originally submitted to the Japanese Patent Office during the prosecution of the Japanese counterpart of the present application, and establishes that restenosis and reendothelization are two independent events that are affected differently by different compounds, and that it cannot be predicted whether an agent known to prevent or reduce smooth muscle cell proliferation and/or to prevent or reduce blood vessel wall thickening will also promote reendothelization (Dec. at, e.g., ¶¶ 12-14). O'Brien is silent about the effect of 17- $\beta$  estradiol on improving reendothelization and vascular endothelial function.

Therefore, the skilled person reading O'Brien would be left with the suggestion that (1) the exact mechanism(s) of action underlying the ability of estrogens to modulate restenosis are unknown; and (2) there is absolutely no expectation of success that administration of estrogens will have an effect on SMCs proliferation in humans, given the fact that this was not observed in nonhuman primates. Therefore, O'Brien fails to

disclose or suggest administering a specific amount of 17- $\beta$  estradiol with a device at an injured site to improve reendothelization and vascular endothelial function following vascular injury, as presently claimed.

The Action also relies on Bauters (Evidence Appendix at 0056-65) as allegedly suggesting the connection between restenosis/SMC proliferation and reendothelization. Bauters discloses that endothelial cells may maintain SMC quiescence through the growth inhibitory effect of NO and that dysfunctional regenerating endothelium in which NO production is compromised may contribute to the development of a thickened intima because of reduced inhibition of platelet aggregation and SMC proliferation. Importantly, Bauters also teaches that restenosis is clearly a multifactorial entity whose pathogenesis is caused by a combination of factors including SMC proliferation, elaboration of extracellular matrix (ECM), thrombosis and vascular remodelling. Bauters also discloses that the contribution of these individual elements does not appear to be consistently proportional from one patient to another, or even one lesion to another in the same patient. (see page 113, left column). Therefore, upon a fair reading of Bauters, the skilled person would conclude (1) that the potential factors, or combination of factors, contributing to restenosis cannot be determined precisely and may vary from one subject to another, or even one lesion to another in the same subject, and (2) that SMC proliferation, one of these alleged factors, may be influenced by the status of the endothelium. Appellant respectfully submits that someone considering the teaching of Bauters would not be left with the suggestion that administration of a specific amount of 17- $\beta$  estradiol with a device at an injured site

would improve reendothelialization and vascular endothelial function following vascular injury, as presently claimed.

In view of the foregoing, Appellant respectfully submits that the Office has failed to establish a *prima facie* case of obviousness.

Appellant also respectfully submits that *ex post facto* analysis of the invention and *ex post facto* synthesis of an artificially contrived mosaic of documents is improper to establish obviousness. The Office, starting from the present application as a guide through the prior art references, specifically chooses and combines bit and pieces from different references, irrespective of the general teaching of these references, in a way to try to arrive at the claimed invention, which is impermissible. "[O]ne cannot simply backtrack from the invention to find a connection to the prior art. Hindsight must be avoided." (*Nursery Supplies Inc. v. Lerio Corp.*, 45 USPQ2d 1332, 1334 (M.D. Pa. 1997), citing *W.L. Gore and Assoc., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 220 USPQ 303 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851 (1984)). Furthermore, the Federal Circuit has stated that "selective hindsight is no more applicable to the design of experiments than it is to the combination of prior art teachings. There must be a reason or suggestion in the art for selecting the procedure used, other than the knowledge learned from the applicant's disclosure." *In re Dow Chemical Co.* 837 F.2d 469, 473, 5 U.S.P.Q. 2d (BNA) 1529, 1532 (Fed Cir. 1988).

It is clear from the teaching of O'Brien that there is absolutely no expectation of success that administration of estrogens will have an effect on SMCs proliferation in humans, given the fact that this was not observed in nonhuman primates. In fact, the results obtained in nonhuman primates teach away from using estrogens to inhibit

SMCs proliferation in humans. Furthermore, it is also clear from the teaching of Bauters that the potential factors, or combination of factors, contributing to restenosis cannot be determined precisely and may vary from one subject to another, or even one lesion to another in the same subject and that SMC proliferation, one of these alleged factors, may be influenced by the status of the endothelium. It appears that the Office is applying an improper "obvious to try" rationale in support of the obviousness rejection. See *In re O'Farrell*, 853 F.2d 894, 7 U.S.P.Q. 2d (BNA) 1673 (Fed Cir. 1988), in which the Federal Circuit acknowledged that it is improper to reject a claim when what was "obvious to try" would have been to vary all parameters or try each of the numerous possible choices until one possibly arrived at the successful results, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful. Again, the expectation of success must be objectively determined from the perspective of a person or ordinary skilled in the art (i.e. not that of the inventors) at the time the invention was made, and importantly this determination must not be influenced or biased by hindsight, notably by the fact that the inventors were successful. Appellant respectfully submits that there was no reasonable expectation of success, at the time the invention was made, that administration of a specific amount of estradiol with a device at an injured site of a blood vessel would improve reendothelialization and vascular endothelial function.

Appellant respectfully submits that Ungs, O'Brien *et al.* and Bauters *et al.*, taken alone or in combination, fail to teach all the elements of claim 1, and thus that a *prima facie* case of obviousness has not been established. None of these references discloses or suggests the administration of 17- $\beta$  estradiol or a derivative thereof in an

amount effective to improve reendothelization and vascular endothelial function, as presently claimed. Further, these references are also silent about the 17- $\beta$  estradiol or derivative thereof being present in a dose unit of 1 to 5000  $\mu$ g/Kg of patient's body, as recited in instant claim 1.

The Office alleges that Unga teaches administering the same compound via the same method as that instantly claimed, and to reduce the incidence of restenosis following a treatment such as PTCA that induces vascular injury, it is considered that the method of Unga would necessarily also improve reendothelization and vascular endothelial function in a patient having vascular suffered vascular injury. Appellant respectfully disagrees and submits that it is improper to assume that the method disclosed would also improve reendothelization and vascular endothelial function. Inherency cannot be based on mere probabilities or possibilities. "The mere absence [from the reference] of an explicit requirement [of the claim] cannot reasonably be construed as an affirmative statement that [the requirement is in the reference]". *In re George R. Evanega and Winfried Albert*, 829 F.2d 1110, 1112, 4 USPQ2d 1249, 1251 (Fed. Cir. 1987). Furthermore, that a certain result or characteristic may occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic. A *prima facie* case of unpatentability is not established merely by speculation that claim elements are "probably satisfied" by the prior art. *In re Rijckaert*, 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993). Inherency may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient. *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999). "In relying upon the theory of inherency, the

Examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art." *Ex parte Levy*, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990).

It is well known that the biological effects of an agent vary considerably with the dose administered, sometimes over several orders of magnitude. The present inventors have shown that administration of 17- $\beta$  estradiol in a dose unit of 1 to 5000  $\mu\text{g/Kg}$  of the patient's body results in improved reendothelization and vascular endothelial function. Unga discloses that estrogens may be used to induce angiogenesis, but does not provide any indication of the dose that is effective to achieve this biological effect. It is possible that the doses of estrogens that induce angiogenesis are different than those which improve reendothelization and vascular endothelial function, and there is no indication that the method taught by Unga would improve reendothelization and vascular endothelial function in a patient having suffered vascular injury.

In regards to the amount of 17- $\beta$  estradiol, the Office mentions that it is considered that one of ordinary skill in the art would have found it obvious to vary and/or optimize the amount of 17- $\beta$  estradiol provided in the method, according to the guidance provided by Unga, to provide the desired treatment, such as the desired reduction in restenosis. Appellant respectfully disagrees and submits that Unga fails to provide any guidance with respect to the dose of 17- $\beta$  estradiol that may be administered. Furthermore, it is submitted that Unga is concerned with a different biological effect, namely inducing angiogenesis, and thus even if Unga would have provided any guidance with respect of the amount of estradiol (which is clearly not the

case), it would be totally irrelevant to the method of the present invention which aims at improving reendothelization and vascular endothelial function.

The Office further refers to *In re Aller*, 220 F. 2d 454, 456, 105 USPQ 233, 235 (CCPA 1955), where it was stated: "[W]here the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experiments". Appellant respectfully submits that *Aller* is inapplicable. First, in *Aller*, the claimed process and the process of the prior art were for the same purpose, namely to produce phenol from an organic peroxide. In contrast, the present claims are directed to the improvement of reendothelization and vascular endothelial function, whereas Ungs relates to the induction of angiogenesis. Secondly, in the prior art considered in *Aller*, specific values were disclosed with respect to the conditions (namely the temperature and the concentration of sulphuric acid) under which the process could be performed, and the claimed process only recited slightly different values for these two parameters (i.e. lower temperatures and higher concentrations of sulphuric acid). In contrast, Ungs fails to disclose or suggest any amount of 17- $\beta$  estradiol that may be administered, and thus fails to disclose or suggest the "general conditions of the claim". Furthermore, as mentioned above, even if Ungs had provided any guidance with respect of the amount of estradiol that may be administered (which is not the case), it would be irrelevant to the presently claimed method, which has a different purpose than the method of Ungs.

**3. The rejection of claims 5-7, 11 and 21 under 35 U.S.C. § 103(a) as being allegedly obvious over the combination of U.S. Patent No. 5,866,561 to Mark T. Ungs ("Ungs"), O'Brien, Bauters *et al.* and Pitha (US Patent No. 4,727,064) is improper and should be reversed.**

Ungs, O'Brien *et al.* and Bauters *et al.* have been discussed in detail above, and for the same reasons do not make out a *prima facie* case of obviousness with respect to claims 5-7, 11 and 21. Further, Pitha (Evidence Appendix at 0066-73) does not remedy the defects in Ungs, O'Brien *et al.* and Bauters *et al.* Indeed, Pitha is relied on merely as suggesting that hydroxypropyl-beta-cyclodextrin may be used to solubilize estradiol. However, it is respectfully submitted that Pitha is not combinable with Ungs. While Pitha does discuss solubilizing estradiol in hydroxypropyl beta-cyclodextrin, that is in the context of preparing suitable topical, parenteral, oral or buccal preparations (see col. 2, lines 45-61). It says nothing about the applicability of its teachings in the context of a device (e.g., for PTCA or coated stents). One of ordinary skill working with drug application via PTCA would not look to Pitha, or be led by it or the combination of references to the inventions of claims 5-7, 11 and 21.

**4. The rejection of claims 1, 3-4, 8, 10-14, 16-18 and 20-24 under 35 U.S.C. § 103(a) as being allegedly obvious over the combination of U.S. Patent No. 5,866,561 to Mark T. Ungs ("Ungs"), Fontana (US 5,384,332) and Grainger *et al.* (US 6,117,911) is improper and should be reversed.**

Ungs has been discussed in detail above, and for the same reasons does not make out a *prima facie* case of obviousness with respect to claims 1, 3-4, 8, 10-14, 16-18 and 20-24. Appellant respectfully submits that Fontana (Evidence Appendix at 0074-79) and Grainger (Evidence Appendix at 0080-134) do not remedy the defects in Ungs.

Fontana teaches a method for inhibition of aortal smooth muscle cell proliferation and restenosis using 1,1,2-triphenylbut-1-ene derivatives in humans. Fontana teaches that the compound can be administered through the oral, rectal, transdermal, intramuscular or intranasal route (col. 5, lines 28-31). Finally, the teachings of Fontana regarding the amount of estradiol (i.e. 0.1 to 99.9% by weight of the formulation) is irrelevant, since it relates specifically to the formulation of the medicament and not to the amount administered to the patient. Also Fontana refers to the amount of 1,1,2-triphenylbut-1-ene derivatives (not the amount of 17- $\beta$  estradiol) and is concerned with oral, rectal, transdermal, intramuscular or intranasal formulations. Appellant respectfully submits that there is nothing in Fontana that teaches or suggests that the administration of a specific amount of 17- $\beta$  estradiol with a device at an injured site would improve reendothelization and vascular endothelial function following vascular injury, as presently claimed.

Appellant respectfully submits that Grainger *et al.* does not render the present invention obvious. Grainger *et al.* teaches a method of treating a mammal having, or at risk of, an indication associated with a TGF-beta deficiency (e.g., atherosclerosis) comprising administering one or more agents that is effective to elevate the level of TGF-beta, more particularly an aspirinate. Appellant respectfully submits that aspirinates and estrogens (e.g., 17- $\beta$  estradiol) have totally different structures and belong to different categories of compounds, and thus the fact that Grainger teaches that aspirinates have an effect on SMCs proliferation and reendothelization is irrelevant to the present invention, which relates to 17- $\beta$  estradiol. There is nothing in Grainger that teaches or suggests that the administration of a specific amount of 17- $\beta$  estradiol

with a device at an injured site would improve reendothelization and vascular endothelial function following vascular injury, as presently claimed.

Therefore, neither Fontana nor Grainger et al. suggests that administration of a specific amount of 17- $\beta$  estradiol with a device at an injured site would improve reendothelization and vascular endothelial function following vascular injury, as presently claimed. As mentioned above, Ungs is also silent about the improvement of reendothelization and vascular endothelial function by administration of 17- $\beta$  estradiol at the injured site of a vessel. Also, none of these references discloses or suggests the administration of 17- $\beta$  estradiol or a derivative thereof in an amount effective to improve reendothelization and vascular endothelial function, as presently claimed. Further, these references are also silent about the 17- $\beta$  estradiol or derivative thereof being present in a dose unit of 1 to 5000  $\mu$ g/Kg of the patient's body, as recited in instant claim 1. The lack of teaching of Ungs with respect to the amount of 17- $\beta$  estradiol has been discussed in detail above. The teaching of Fontana regarding the amount of estradiol (i.e. 0.1 to 99.9% by weight of the formulation) is irrelevant, since it relates specifically to the formulation of the medicament and not to the amount administered to the patient. Also Fontana refers to the amount of 1,1,2-triphenylbut-1-ene derivatives (not the amount of 17- $\beta$  estradiol) and is concerned with oral, rectal, transdermal, intramuscular or intranasal formulations.

In view of the foregoing, Appellant respectfully submits that Ungs, Fontana and Grainger et al., taken alone or in combination, fail to teach all the elements of claims 1, 3-4, 8, 10-14, 16-18 and 20-24, and thus that a *prima facie* case of obviousness has not been established.

5. The rejection of claims 5-7 under 35 U.S.C. § 103(a) as being allegedly obvious over the combination of U.S. Patent No. 5,866,561 to Mark T. Ungs ("Ungs"), Fontana (US 5,384,332), Grainger *et al.* (US 6,117,911) and Pitha (US Patent No. 4,727,064) is improper and should be reversed.

Ungs, Fontana and Grainger *et al.* have been discussed in detail above, and for the same reasons do not make out a *prima facie* case of obviousness with respect to claims 5-7. Further, Pitha does not remedy the defects in Ungs, Fontana and Grainger *et al.* Indeed, Pitha is relied on merely as suggesting that hydroxypropyl-beta-cyclodextrin may be used to solubilize estradiol. However, it is respectfully submitted that Pitha is not combinable with Ungs. While Pitha does discuss solubilizing estradiol in hydroxypropyl beta-cyclodextrin, that is in the context of preparing suitable topical, parenteral, oral or buccal preparations (see col. 2, lines 45-61), it says nothing about the applicability of its teachings in the context of a device (e.g., for PTCA or coated stents). One of ordinary skill working with drug application via PTCA would not look to Pitha, or be led by it or the combination of references to the inventions of claims 5-7.

### Conclusion

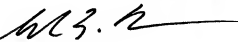
Appellant respectfully requests that the rejections of claims 1, 3-8, 10-14, 16-18 and 20-24 be reversed.

Respectfully submitted,

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Dated: June 5, 2008

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**(viii) Claims Appendix**

1. A method of improving reendothelization and vascular endothelial function in a patient in need of such improvement, which comprises administering to said patient 17- $\beta$  estradiol or a derivative thereof, in an amount effective to improve reendothelization and vascular endothelial function, with a device at an injured site in the lumen of a blood vessel having suffered vascular injury, wherein the 17- $\beta$  estradiol or a derivative thereof is present in a dose unit of 1 to 5000  $\mu\text{g/Kg}$  of patient's body weight.

3. The method of claim 1, wherein the 17- $\beta$  estradiol or a derivative thereof is present in a dose unit of 10 to 50  $\mu\text{g/Kg}$  of patient's body weight.

4. The method of claim 1, wherein the 17- $\beta$  estradiol or a derivative thereof is present in a dose unit of 10 to 30  $\mu\text{g/Kg}$  of patient's body weight.

5. The method of claim 1, wherein the 17- $\beta$  estradiol or derivative thereof is present in a pharmaceutically acceptable carrier wherein said pharmaceutically acceptable carrier comprises hydroxypropyl-beta-cyclodextrin (HPCD).

6. The method of claim 5, wherein the HPCD is present in an amount capable of solubilizing the 17- $\beta$  estradiol or a derivative thereof.

7. The method of claim 4, wherein said 17- $\beta$  estradiol or a derivative thereof is administered admixed with a carrier comprising at least 0.63 mg hydroxypropyl beta cyclodextrin per kilogram of patient's body weight.

8. The method of claim 1, which comprises a single administration.

10. The method of claim 1, wherein the device is a catheter containing said 17- $\beta$  estradiol or derivative thereof, and a pharmaceutically acceptable carrier.

11. The method of claim 1, wherein the device is a stent coated with said 17- $\beta$  estradiol or derivative thereof, and a pharmaceutically acceptable carrier.

12. The method of claim 1, wherein said 17- $\beta$  estradiol or derivative thereof is administered following percutaneous transluminal coronary angioplasty (PTCA).

13. The method of claim 1, wherein said 17- $\beta$  estradiol or derivative thereof is administered simultaneously to percutaneous transluminal coronary angioplasty (PTCA).

14. The method of claim 1, wherein said method uses 17- $\beta$  estradiol.

16. The method of claim 14, wherein the 17- $\beta$  estradiol is present in a dose unit of 10 to 50  $\mu\text{g/Kg}$  of patient's body weight.

17. The method of claim 14, wherein the 17- $\beta$  estradiol is present in a dose unit of 10 to 30  $\mu\text{g/Kg}$  of patient's body weight.

18. The method of claim 14, which comprises a single administration.

20. The method of claim 14, wherein the device is a catheter containing said 17- $\beta$  estradiol, and a pharmaceutically acceptable carrier.

21. The method of claim 14, wherein the device is a stent coated with said 17- $\beta$  estradiol, and a pharmaceutically acceptable carrier.

22. The method of claim 14, wherein said 17- $\beta$  estradiol is administered following percutaneous transluminal coronary angioplasty (PTCA).

23. The method of claim 14, wherein said 17- $\beta$  estradiol is administered simultaneously to percutaneous transluminal coronary angioplasty (PTCA).

24. The method of claim 1, wherein said 17- $\beta$  estradiol or derivative thereof is the only therapeutic moiety administered.

**(ix) Evidence appendix**

1.	Declaration by Dr. Richard Stack and his Resume, filed January 23, 2006.....	0001
2.	Ungs U.S. Patent No. 5,866,561 cited by Examiner.....	0044
3.	O'Brien reference cited by Examiner.....	0048
4.	Bauters reference cited by Examiner.....	0056
5.	Pitha U.S. Patent No. 4,727,064 cited by Examiner.....	0066
6.	Fontana U.S. Patent No. 5,384,332 cited by Examiner.....	0074
7.	Grainger U.S. Patent No. 6,117,911 cited by Examiner.....	0080
8.	Hughes U.S. Patent No. 5,516,528 discussed in the Amendment filed June 5, 2007.....	0135
9.	Javitt U.S. Patent No. 5,376,652 discussed in the Amendment filed June 5, 2007.....	0146

I hereby certify that this correspondence is being deposited with the United States Postal Service on the date set forth below as First Class Mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.  
Date of Signature \_\_\_\_\_  
and Deposit: \_\_\_\_\_

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicants:	Institut de Cardiologie de Montréal
Serial No.:	2001-5324583
Filed:	September 21, 2000
For:	LOCAL DELIVERY OF 17-BETA ESTRADIOL FOR PREVENTING VASCULAR INTIMA HYPERPLASIA AND FOR IMPROVING VASCULAR ENDOTHELIAL FUNCTION AFTER VASCULAR INJURY
Examiner:	Hidenori ARAKI

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**DECLARATION**

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Dear Sir:

1. I, Richard Sean Stack, M.D., FACC, residing at 106 Alder Place, Chapel Hill, NC 27517, am Professor Emeritus of Cardiology at Duke University Medical Center, Erwin Rd., Durham, NC. 27710
2. I have been conducting research in the field of angioplasty and restenosis for 26 years and have published more than 350 scientific publications on the subject. I have numerous close associates in the Japanese Cardiology Community and have appeared as Guest of Honor at many Japanese national meetings such as the Japanese Coronary Association. Please find enclosed a copy of my resume.

Blood vessels, endothelium and smooth muscle cells

3. All blood vessels follow a similar histological makeup in the arterial system. The inner lining is the endothelium, followed by subendothelial connective tissue. Then follows a muscular layer of vascular smooth muscle. Finally, there is a further layer of connective tissue termed the adventitia, which contains nerves that supply the muscular layer, as well

as nutrient capillaries in the larger blood vessel. The amount of collagen and elastic fibers within the vessel wall can vary with the size of a given vessel.

4. The endothelium is a layer of thin, flat cells, i.e. endothelial cells, that lines the interior surface of blood vessels, forming an interface between circulating blood in the lumen and the rest of the vessel wall.
5. Endothelial cells are distinct by their function and by their nature from smooth muscle cells. Smooth muscle cells are responsible for the contraction and dilatation of blood vessels. Endothelial are characterized by their permeability and thus assist in absorbing nutrients from the blood and releasing them in surrounding tissues. Endothelial cells also play a role in inflammation and are responsible for the release of a vasodilator, namely nitric oxide and other anti-thrombotic substances like tissue-plasminogen activator.

#### Restenosis

6. When an abnormal narrowing of a blood vessel's channel (i.e. stenosis), occurs, it may be necessary to insert a device in the vessel to increase the channel's size. This revascularization technique is called angioplasty. During angioplasty, the blood vessel may be injured by the insertion of the device. The injury to the vessel generally consists in scraping away the first layers of the blood vessel wall including the innermost layer, the endothelium and variable tearing of the atherosclerotic wall.
7. This injury may then lead to a "restenosis", namely a re-obstruction of the blood vessel channel. This re-obstruction is the result of a number of events including pathological proliferation and migration of smooth muscle cells which cause thickening of the blood vessel wall and a narrowing of the blood vessel wall.

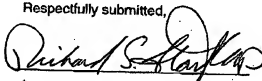
Prevention of restenosis

8. In order to prevent restenosis, it is thus useful to inhibit smooth muscle cell proliferation, namely prevent smooth muscle cells to multiply.
9. More recently, it was found however that in order to prevent restenosis, it is also useful to promote vessel repair, namely promote the regeneration of the endothelium and consequently a restoration of the endothelial function such as nitric oxide release. The regeneration of endothelium is also called "reendothelialization".
10. There are a number of agents known to prevent or reduce smooth muscle cell proliferation and/or to prevent or reduce blood vessel wall thickening. In fact most anti-restenosis agent are anti-proliferative agents that seek to prevent proliferation of smooth muscle cells.
11. Very few agents are known to promote blood vessel wall repair. In fact, until recently, very few appreciated the usefulness of regenerating the endothelium. On the contrary, many believed in a toxic approach such as brachytherapy to kill smooth muscle cells and avoid the endothelium to repair.
12. It is my experience that it cannot be predicted whether an agent known to prevent or reduce smooth muscle cell proliferation and/or to prevent or reduce blood vessel wall thickening will also promote reendothelialization.
13. Indeed, substances able to inhibit smooth muscle cell proliferation or migration do not systematically also improve reendothelialization or vascular function. Hence paclitaxel and sirolimus are anti-proliferative agents that do not promote reendothelialization.
14. In my opinion therefore, the knowledge that beta-estradiol had an ability to reduce smooth muscle cell proliferation was not sufficient, for someone

skilled in the art, to predict that beta-estradiol could also promote reendothelization and endothelial function.

15. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true.

Respectfully submitted,



7-25-05

Date

## CURRICULUM VITAE

**RICHARD SEAN STACK, M.D., F.A.C.C.**

### CONTACT INFORMATION:

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DATE OF BIRTH: May 14, 1949 (Detroit, Michigan)

### MEDICAL EDUCATION:

1972-1976	Wayne State University Medical School, Detroit, Michigan
1976-1977	Internship (Internal Medicine), Harper Hospital, Wayne State University, Detroit, Michigan
1977-1978	Residency (Internal Medicine), Harper Hospital, Wayne State University, Detroit, Michigan
1978-1979	Chief Resident (Internal Medicine), Harper Hospital, Wayne State University, Detroit, Michigan
1979-1982	Fellowship (Cardiology), Duke University Medical Center, Durham, North Carolina 27710

## CURRICULUM VITAE

RICHARD SEAN STACK, M.D.

### MEDICAL POSITIONS AND POSTS:

1982-1983	Instructor in Medicine (Cardiology), Duke University Medical Center, Durham, North Carolina 27710
1982-1984	Senior Staff Physician, Director, Coronary Care Unit, Durham VA Medical Center, Durham, North Carolina 27710
1983-1989	Assistant Professor of Medicine (Cardiology), Duke University Medical Center, Durham, North Carolina 27710
1983-2002	Founder and Director of Interventional Cardiology, Duke University Medical Center, Durham, North Carolina 27710
1989-1997	Associate Professor of Medicine (Cardiology, tenured), Duke University Medical Center, Durham, North Carolina 27710
1997-2002	Professor of Medicine (Cardiology, tenured), Duke University Medical Center, Durham, North Carolina 27710
2002-Present	Professor Emeritus, Dept. of Medicine (Cardiology)

### GRANTS AND AWARDS:

1. Wayne State University Research award for best medical student research - 1976.
2. Duke University Inman award for best basic science research in cardiology - 1980.
3. GRANT: Coronary Blood Flow Velocity in Ischemic Heart Disease NHLB1 No. R01 HL37608-01 - Active 1987-1990, Principal Investigator
4. GRANT: Bypass Angioplasty Revascularization Investigation (BARI) Clinical Trial NHLB1 NO. U01 HL38516-01 - Active 1987-1991, Co-principal Investigator
5. GRANT: Development of Peripheral and Coronary Vascular Bioabsorbable Stent Prostheses NHLB1 No. 2 P50 HL17670-16 - Active 1990-1991, Principal Investigator
6. International Award for Best Experimental Research in 1995 presented by Erasmus University Thoraxcenter, Netherlands.
7. Who's Who In America - Cardiology - 1997
8. The Best Doctors in America - Cardiology - 1997
9. Distinguished Alumni Award, Wayne State University School of Medicine 2001
10. Professor Emeritus granted by Duke Board of Trustees
11. "Richard S. Stack, MD Professorship" Permanent Endowed Chair - Duke University

## CURRICULUM VITAE

RICHARD SEAN STACK, M.D.

ORGANIZATIONS:

American Heart Association  
 American College of Cardiology  
 American Medical Association  
 North Carolina Medical Society  
 North Carolina Heart Association  
 Duke Biomedical Engineering Industrial Advisory Board

CERTIFICATIONS:

Board Certification: American Board of Internal Medicine  
 Subspecialty Board Certification: American Board of Internal Medicine, Cardiology  
 Fellow, American College of Cardiology

MEDICAL LICENSURE:

North Carolina  
 California

INTERVENTIONAL DEVICE INVENTIONS: (55 US Patents and Patents Pending)A. Patents Issued: (24 patents)

January 13, 2004	6,675,809	Satiation devices and methods
January 6, 2004	6,673,891	Polymers for delivering nitric oxide in vivo
November 11, 2003	6,645,223	Deployment and recovery control systems for embolic protection devices
July 15, 2003	6,592,616	System and device for minimizing embolic risk during an interventional procedure
April 1, 2003	6,540,722	Embolic protection devices
January 14, 2003	6,506,203	Low profile sheathless embolic protection system
November 19, 2002	6,481,262	Stent crimping tool
October 22, 2002	6,468,299	Stent delivery catheter with bumpers for improved retention of balloon expandable stents
August 20, 2002	6,436,132	Composite intraluminal prostheses
June 11, 2002	6,403,759	Polymers for delivering nitric oxide in vivo
November 27, 2001	6,322,490	Radioactive stent structures
July 24, 2001	6,264,683	Stent delivery catheter with bumpers for improved retention of balloon expandable stents
July 24, 2001	6,264,671	Stent delivery catheter and method of use

## CURRICULUM VITAE

RICHARD SEAN STACK, M.D.

May 15, 2001	6,232,434	Polymers for delivering nitric oxide in vivo
December 26, 2000	6,165,196	Perfusion-occlusion apparatus
October 31, 2000	6,139,511	Guidewire with variable coil configuration
June 23, 1998	5,770,645	Polymers for delivering nitric oxide in vivo
June 18, 1996	5,527,337	Bioabsorbable stent and method of making the same
August 30, 1994	5,342,393	Method and device for vascular repair
April 26, 1994	5,306,286	Absorbable stent
October 22, 1991	5,059,211	Absorbable vascular stent
March 6, 1990	4,905,689	Method of using a laser catheter
September 19, 1989	4,867,156	Percutaneous axial atherectomy catheter assembly and method of using the same
August 8, 1989	4,854,315	Laser catheter

## B. Patents Pending and Published by US Patent Office: 19 Patent Applications

Filed Date	Application Number	
September 15, 2003	20040088002	Deployment and recovery control systems for embolic protection devices
June 9, 2003	20030199991	Satiation devices and methods
June 9, 2003	20030199990	Satiation devices and methods
June 9, 2003	20030199989	Satiation devices and methods
May 29, 2003	20030195556	System and device for minimizing embolic risk during an interventional procedure
March 10, 2003	20030212361	Embolic protection devices
January 16, 2003	20040117031	Satiation devices and methods
June 5, 2002	20030078365	Novel polymers for delivering nitric oxide in vivo
April 8, 2002	20030040808	Satiation devices and methods
August 27, 2001	20030040804	Satiation devices and methods
August 13, 2001	20030032941	Convertible delivery systems for medical devices
July 31, 2001	20010051822	Self-expanding stent with enhanced delivery precision and stent delivery system
June 20, 2001	20020124626	Stent crimping tool
June 19, 2001	20010037126	Stent delivery catheter and method of use

**CURRICULUM VITAE****RICHARD SEAN STACK, M.D.**

June 7, 2001	20010044630	Stent delivery catheter with bumpers for improved retention of balloon expandable stents
June 7, 2001	20010044630	Stent delivery catheter with bumpers for improved retention of balloon expandable stents
April 30, 2001	20020161389	Deployment and recovery control systems for embolic protection devices
December 29, 2000	20010020083	Novel Polymers for Delivering nitric oxide in vivo
December 22, 2000	20020042625	Perfusion-occlusion apparatus and methods

C. Patents Pending - Submitted to Patent Office and not yet published (less than 18 months since submission): 12 Patent Applications

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**EXTRACURRICULAR ACTIVITIES:**

- Martial arts: 6<sup>TH</sup> Degree Black Belt: To Shin Do  
9<sup>th</sup> Degree Black Belt: Ninjustsu

**SCIENTIFIC PUBLICATIONS: 333 Manuscripts, Chapters, Books, and Abstracts**

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**LETTERS TO THE EDITOR**

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US005866561A

**United States Patent** [19][11] **Patent Number:** 5,866,561

Ungs

[45] **Date of Patent:** Feb. 2, 1999[54] **LOCAL DELIVERY OF ESTROGEN FOR ANGIOGENESIS**5,580,722 12/1996 Foulkes et al. 435/6  
5,591,129 1/1997 Shoup et al. 604/96[75] **Inventor:** Mark T. Ungs, St. Paul, Minn.**FOREIGN PATENT DOCUMENTS**[73] **Assignee:** SciMed Life Systems, Inc., Maple Grove, Minn.

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[51] **Int. Cl.<sup>6</sup>** ..... A61K 31/56

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[52] **U.S. Cl.** ..... 514/182; 604/96

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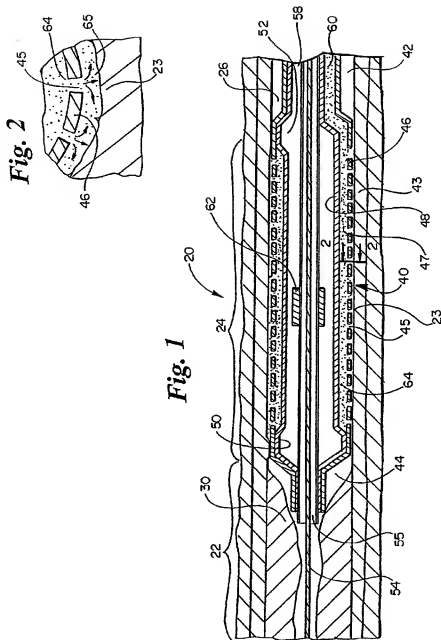
**Primary Examiner**—Leonard Schenkman**Attorney, Agent, or Firm**—Crompton, Seager & Tufte, LLC

[57]

**ABSTRACT**

A method for inducing angiogenesis in blood vessels proximal to ischemic tissue or proximal to stenosed regions including application of an estrogen compound to the blood vessel walls at a treatment site proximal to or upstream of the stenosis. A preferred delivery device is a double walled drug delivery catheter having porous outer walls. Another suitable delivery device is a drug injection device for injecting angiogenic material into blood vessel walls. One delivery method utilizes iontophoresis.

**9 Claims, 1 Drawing Sheet**



# 1 LOCAL DELIVERY OF ESTROGEN FOR ANGIOGENESIS

## FIELD OF THE INVENTION

The invention relates generally to a method for inducing angiogenesis upstream of blood vessel occlusions. More specifically, the invention relates to applying estrogen compounds to blood vessel regions to induce angiogenesis upstream of ischemic tissue.

## BACKGROUND OF THE INVENTION

Atherosclerosis and the resulting myocardial infarction is a leading cause of death, particularly in males. Percutaneous Transluminal Coronary Angioplasty (PTCA) is one treatment used to treat patients with coronary artery disease. PTCA can relieve myocardial ischemia by dilating lumen obstructions, thereby increasing coronary blood flow. Unfortunately, restenosis following PTCA is a significant problem, occurring about 30% of the time within 6 months. Various treatments have been suggested to deal with restenosis.

Administration of compounds for inhibiting vascular smooth muscle cell proliferation and restenosis has been suggested by Cullinan et al. (U.S. Pat. No. 5,462,937). Woods (U.S. Pat. No. 5,180,366) discloses an application of smooth muscle cell anti-proliferation agents, including estrogen, and endothelial growth factor to inhibit restenosis has also been suggested. Growth and division of endothelial cells was said to be promoted while proliferation of smooth muscle cells was believed to be inhibited.

Application of Vascular Endothelial Growth Factor (VEGF) as a post-operative wound healing agent after balloon angioplasty has been suggested, in an amount effective to promote endothelial cell growth by Tischer et al. (U.S. Pat. No. 5,219,739). Inhibition of smooth muscle cell proliferation by administration of an effective amount of Transforming Growth Factor-beta activators or production simulators that act to inhibit vascular smooth muscle cell proliferation has also been proposed by Grainger et al. (U.S. Pat. No. 5,472,985).

It is believed that the more common occurrence of restenosis in men compared to women suggests hormones play a role. Oral, transdermal, and implant delivery administration of a therapeutically effective amount of estrogen has been suggested as a method for reducing the risk of heart disease by Hughes et al. (U.S. Pat. No. 5,516,528). A method for reducing restenosis by administering estrogen in a dose sufficient to stimulate synthesis of 27-hydroxycholesterol in the vascular endothelium tissue has also been proposed by Javitt (U.S. Pat. No. 5,376,652). Administration of estrogen to the stenosed, dilated region after PTCA has thus been suggested for the purposes of preventing restenosis.

PTCA is not always a successful solution or even a viable treatment option, as not all stenosed regions can be treated with PTCA. For example, some regions are unreachable with the required high pressure balloon, which must be advanced through the narrowed, occluded vessel region. Some stenoses are totally blocked, denying entry to a dilation catheter attempting to advance within. Other vessel regions are too narrow or geometrically too tortuous for dilation. Damage to weakened vessel walls is a possibility during balloon inflation as well, and may preclude PTCA in some cases. Treatment to increase perfusion to heart tissue, in place of, or in addition to, PTCA would be desirable.

## SUMMARY OF THE INVENTION

The present invention provides a method for increasing circulation to heart tissue involving the application of estrogen compounds to blood vessel walls to promote angiogenesis. Applicants believe estrogen induces angiogenesis (blood vessel growth) and increases permeability. This provides increased local blood circulation through neovascularization, the creation of new blood vessels.

A preferred location for application of the estrogen is proximal to, or upstream of, ischemic tissue or a stenosis. This creates new blood vessels proximal to the ischemic tissue or a stenosis. The invention is relatively more beneficial when practiced in smaller blood vessels when it is considered that smaller vessels are the vessels more likely to present difficulties in being crossed with balloon catheters and dilated, while also being the vessels which supply blood to a smaller area of tissue which is believed benefited most from angiogenesis.

One method of delivering the angiogenesis inducing compound or estrogen compound includes application with a double walled drug delivery balloon catheter having a porous outer wall. A preferred catheter is a perfusion balloon catheter. The balloon can be advanced to a site proximal the ischemic tissue or stenosis, the inner balloon inflated, bringing the outer balloon into close contact with the blood vessel wall. The compound can then be injected into the catheter lumen, thereafter flowing into the space between the balloon walls, and out to contact the vessel walls.

In yet another embodiment, a balloon envelope is coated with a viscous or otherwise difficult to inject estrogen containing compound. The balloon envelope and a sheath are advanced co-extensively until reaching the treatment site, the sheath withdrawn, and the balloon expanded, forcing the compound against the vessel wall.

Another method of delivering the estrogen compound includes use of iontophoresis. A delivery balloon releases the compound within the vessel, where the compound is ionic or can be carried along with an ionic material. Electrodes external to the patient's body are used to create an electric field which acts as a driving force to cause molecules to advance toward an oppositely charged pole.

Yet another method of compound delivery includes advancing a drug delivery catheter having a puncturing element to the delivery site. The puncturing element is moved to puncturing position, the inner wall punctured, and the compound injected into the vessel wall. An estrogen compound could also be coated on a stent and placed at a desired delivery site, temporarily or permanently.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a fragmentary side elevational view of a stenosed vessel sectioned vertically on the vessel longitudinal axis and having an estrogen compound applying catheter proximally thereof; and

FIG. 2 is an enlarged sectional view of FIG. 1 taken along 2-2, illustrating estrogen compound flow from application device through outer envelope to the vessel wall.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A preferred method of estrogen compound material delivery includes inflating a balloon having an estrogen compound covering a substantial portion of the balloon. The compound is thus held in place against the vessel wall, promoting adsorption through the vessel wall. A preferred catheter for delivery is a perfusion balloon catheter. A catheter allowing perfusion therethrough allows holding the estrogen compound against the vessel walls for longer

adsorption times while allowing blood to flow through the blood vessel. Examples of catheters suitable for estrogen compound application are drug delivery catheters as disclosed in U.S. Pat. No. 5,558,647, entitled "Drug Delivery Catheter" or U.S. Pat. No. 5,554,119, entitled "Drug Delivery Catheter with Manifold", the disclosures of which are incorporated herein by reference. Another suitable catheter is disclosed in U.S. patent application Ser. No. 08/441,618, filed May 15, 1995, entitled "Perfusion Balloon Angioplasty Catheter", now U.S. Pat. No. 5,591,129, to the present assignee, the disclosure of which is incorporated herein by reference. This disclosed catheter can be constructed with a porous drug delivery member over the balloon, as illustrated in FIG. 1.

FIG. 1 illustrates a blood vessel 20 having an inner wall 23, a lumen 26 and stenosed region 30 and a more proximal region 24, where stenosed region 22 is more occluded and proximal region 24 is less occluded. Stenosed region 22 includes a stenosis 33 within as representative of an area of ischemic tissue. A compound delivery device 40 is illustrated, here double walled balloon catheter 40, inserted to a treatment site in vessel lumen 26 proximal to or upstream of stenosed region 22. Catheter 40 has a balloon 43 extending from balloon proximal end 42 to balloon distal end 44. Balloon 43 includes an inner envelope 48 and an outer, porous envelope 46. An interior space 47 lies between inner envelope 48 and outer envelope 46. The embodiment illustrated includes a proximal protrusion 52 and a distal protrusion 50, both shown extending in close proximity to vessel inner wall 23. Catheter 40 includes a combination perfusion-guide wire lumen 55.

Catheter 40 is shown containing a guide wire 54 within a guide wire lumen 56. Catheter 40 also includes an inflation lumen 58 and a compound application lumen 60. A preferred embodiment includes a radiopaque marker band 62. An estrogen inducing compound 64 is illustrated within lumen 60, within interior space 47, outside outer envelope 46, and in contact with vessel inner wall 23. Estrogen compound 64 is shown flowing between holes 45 in outer envelope 46 to vessel inner wall 23.

FIG. 2 illustrates in greater detail estrogen compound 64 flowing at 65 through holes 45 in outer envelope 46 to vessel inner wall 23. The contours and spaces between the catheter balloon 43 and the vessel wall 23, along with the thickness of estrogen compound 64 are not drawn to scale in FIGS. 1 and 2, but rather illustrate the application of the present invention.

In use, guide wire 54 can be advanced through the vasculature and just proximal to or through stenosis 30. Delivery catheter 40 may then be threaded onto guide wire 54 and advanced to a position proximal to or upstream of stenosis 30. Once in place, inner balloon 43 can be inflated, causing outer balloon 46 to more closely approach or touch

vessel wall 23. Estrogen compound 64 can then be injected into compound lumen 60, forcing the compound into interior space 47 and through holes 45 in porous outer balloon 46. A preferred method of delivery includes further pressurizing inner balloon 48 to force estrogen compound 64 against vessel inner wall 23. Another preferred method includes injecting compound 64 under pressure while inflation pressure is being applied to balloon 48, applying estrogen compound 64 under pressure against vessel inner wall 23. Preferred estrogen compounds include 17-Beta Estradiol, estradiol (E2) or estriol (E3).

Numerous characteristics and advantages of the invention covered by this document have been set forth in the foregoing description. It will be understood, however, that this disclosure is, in many respects, only illustrative. Changes may be made in details, particularly in matters of shape, size, and arrangement of parts without exceeding the scope of the invention. The inventions' scope is, of course, defined in the language in which the appended claims are expressed.

What is claimed is:

1. A method for increasing blood flow to ischemic tissue proximal to a constricted blood vessel region comprising the step of inducing angiogenesis in a less constricted blood vessel region proximal to said constricted region by applying an effective amount of estrogen compound for causing angiogenesis to said less constricted blood vessel region.
2. The method of claim 1, wherein said method further comprises providing an application device, wherein said application device is a balloon catheter.
3. The method of claim 1, wherein said less constricted blood vessel region has walls and said applying step includes depositing said compound on said walls.
4. The method of claim 1, wherein said applying device is a double walled balloon catheter having a porous outer wall.
5. The method of claim 4, wherein said catheter includes a perfusion lumen.
6. The method of claim 1, wherein said less constricted blood vessel region has walls, said application device is an injection device, and said applying step includes injecting said compound into said walls.
7. The method of claim 1, wherein said estrogen compound is selected from the group consisting of estradiol(E2) and estriol(E3).
8. The method of claim 1, wherein said estrogen compound is 17-Beta Estradiol.
9. The method of claim 1, wherein said less constricted blood vessel region has walls, and said method includes providing an application device including an iontophoresis device and said applying step includes depositing said compound on said walls and transporting said compound through said walls utilizing said iontophoresis device.

\* \* \* \* \*

## Relation Between Estrogen Replacement Therapy and Restenosis After Percutaneous Coronary Interventions

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**Objectives.** We attempted to determine the relation between estrogen replacement therapy and the rate of restenosis after coronary angioplasty and atherectomy.

**Background.** Although estrogen replacement therapy in women has been associated with a reduction in cardiovascular events and improvement in endothelial function, no study has examined whether estrogen reduces restenosis rates after percutaneous coronary interventions.

**Methods.** A total of 204 women enrolled in the Coronary Angioplasty Versus Excisional Atherectomy Trial with angiographic follow-up were contacted, and their menopausal and estrogen replacement status was determined. Late loss in minimal lumen diameter, late loss index, minimal lumen diameter, rate of restenosis >50% and actual percent of stenosis were compared in estrogen users and nonusers by quantitative coronary angiography at 6-month follow-up.

**Results.** Late loss in minimal lumen diameter was significantly less in women using estrogen than in nonusers ( $-0.13$  vs.

$-0.46$  mm,  $p = 0.01$ ). A regression analysis of the determinants of late loss in minimal lumen diameter revealed that estrogen use was the single most important predictor of subsequent late loss ( $F = 13.38$ ,  $p = 0.0006$ ). Formal testing revealed a highly significant interaction between the use of estrogen and intervention (angioplasty or atherectomy). Women undergoing atherectomy who received estrogen had a significantly lower late loss index ( $0.06$  vs.  $-0.63$ ,  $p = 0.002$ ), less late loss ( $0.06$  vs.  $-0.61$  mm,  $p = 0.0006$ ), larger minimal lumen diameter ( $p = 0.044$ ) and lower restenosis rates ( $p = 0.038$  for  $>50\%$  stenosis) than those not using estrogen. In contrast, estrogen had minimal effects on restenosis end points after angioplasty.

**Conclusions.** This study demonstrates the potential for estrogen replacement therapy to reduce angiographic measures of restenosis in postmenopausal women after coronary intervention, particularly in those undergoing atherectomy.

(*J Am Coll Cardiol* 1996;28:1111-18)

Restenosis remains a major limitation to the long-term benefit of percutaneous transluminal coronary angioplasty and directional coronary atherectomy. Several clinical studies (1-4) have reported the rate of angiographically defined late restenosis after angioplasty to be between 30% and 50%. Despite multiple clinical trials evaluating numerous pharmacologic agents (5-12), few drugs to date have shown any potential to significantly reduce the rate of restenosis. However, none of these previous studies examined the value of estrogen replacement

therapy in decreasing the rate of restenosis in selected patients.

Extensive observational data exist detailing an association between estrogen replacement therapy and a reduction in cardiovascular disease in postmenopausal women. Primarily epidemiologic studies have demonstrated a 50% reduction in the relative risk of cardiovascular events in postmenopausal women taking estrogen compared with those not receiving estrogen (13,14). In addition, recent studies (15-27) suggest that estrogen may reduce the progression of existing coronary artery disease in postmenopausal women, favorably modulate the vascular biology of atherosclerotic coronary arteries and limit the proliferation of vascular smooth muscle after endothelial injury.

Given these potential favorable effects of estrogen on coronary vascular biology, we hypothesized that estrogen replacement therapy in postmenopausal women would reduce angiographic measures of restenosis and improve functional status and clinical outcomes after percutaneous coronary interventions. To test this hypothesis, we examined the impact of

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estrogen on restenosis in the Coronary Angioplasty Versus Excisional Atherectomy Trial (CAVEAT I).

### Methods

**Study patients.** A detailed description of the trial has been published previously (28). Between August 1991 and April 1992, 275 women who had given written informed consent in the United States and Europe were randomized to either angioplasty or atherectomy at 35 sites participating in CAVEAT I. Eligibility criteria included the presence of symptomatic ischemic heart disease amenable to treatment with either angioplasty or atherectomy, diseased native coronary vessels with no previous coronary interventions, and  $\geq 60\%$  stenosis on visual assessment. Patients with multivessel disease were eligible, but a single target vessel suitable for either angioplasty or atherectomy was specified before coronary intervention. Although the study protocol considered a residual stenosis of  $<20\%$  optimal, a reduction of lesions to  $\leq 50\%$  diameter stenosis was considered a successful procedure.

For the current study, we included only the 254 women randomized within 32 hospitals participating in CAVEAT I within the United States in order to ensure a consistent method of data collection and subsequent verification.

**Angiographic analysis.** As part of CAVEAT I, the procedural details of angioplasty and atherectomy and the results of cardiac catheterization were collected prospectively. The cineangiograms were quantitatively analyzed at the Cleveland Clinic Foundation by technicians who had no knowledge of intervention assignments or outcome data (28). When multiple views of the target lesion were available, the most severe hemiaxial view of the stenosis without foreshortening was selected for analysis. End-diastolic cine frames from orthogonal views were digitized with a cine-video converter and a computer-assisted edge detection algorithm (29).

A total of 204 (80%) of 254 eligible women in CAVEAT I had angiographic follow-up within 6 months. If a patient underwent repeat catheterization before the scheduled procedure because of a change in clinical status, that angiogram was used to obtain follow-up data. The reasons for failure to obtain angiographic follow-up included refusal to undergo repeat catheterization (27 patients), coronary artery bypass surgery ( $n = 10$ ), death ( $n = 5$ ), intercurrent illness ( $n = 2$ ), lost to follow-up ( $n = 5$ ) and withdrawal from the study ( $n = 1$ ). Of the 50 women without angiographic follow-up, 6 were using estrogen replacement therapy. Of these six, three subsequently had coronary artery bypass surgery and all three refused to undergo repeat catheterization.

**End points.** The primary angiographic end point for the current study was prospectively defined as the mean difference in minimal lumen diameter between the 6-month angiogram and the postprocedure angiogram (late loss) as determined by quantitative angiography. This end point has been used in several previous angiographic restenosis trials (4). Secondary angiographic end points included 1) the late loss index (late loss divided by immediate gain in minimal lumen diameter), 2)

minimal lumen diameter at follow-up, 3) dichotomous rate of restenosis ( $>50\%$  stenosis), and 4) actual percent stenosis at 6-month follow-up.

The clinical end points collected at 1 year included all-cause mortality, myocardial infarction, coronary artery bypass surgery, repeat cardiac catheterization and exertional angina class (defined by Canadian Cardiovascular Society class). A composite 1-year clinical end point was also defined as death, myocardial infarction, coronary artery bypass surgery and nonsurgical coronary intervention in the target artery.

**Determination of estrogen replacement use.** A total of 246 (97%) of 254 women previously enrolled in CAVEAT I from the United States were contacted between March and May 1995. Menopausal status and estrogen replacement status at the time of randomization and at the 6-month and 1-year follow-up were collected by structured telephone interview by one investigator who had no knowledge of outcome data. Of 254 women, 7 could not be contacted by telephone but subsequently responded to a mailed questionnaire. A total of eight women could not be contacted by telephone or mailed questionnaire (six of the eight died after randomization and two were lost to follow-up). For the six women who died after randomization, both medical record review and physician contact were used to determine estrogen replacement status. Nine women were determined to be premenopausal at the time of percutaneous coronary intervention by interview or questionnaire and were removed from all subsequent analyses. For the purpose of the study, positive estrogen replacement status was specified as unopposed estrogen or combined estrogen/progestin regimens initiated before the time of initial coronary intervention and used continuously through both 6-month and 1-year follow-up. Any patient who discontinued estrogen replacement therapy before angiographic or 1-year follow-up was considered to be in the nonestrogen replacement group (four patients). Positive estrogen replacement status was verified for all respondents by a combination of medical record review and prescribing physician contact.

**Statistical analysis.** Baseline characteristics of the estrogen replacement and the nonestrogen replacement groups are presented as medians with interquartile ranges for continuous variables and percentages for discrete variables. Selected baseline characteristics and clinical and angiographic outcomes were compared between groups by using the Wilcoxon rank-sum test for continuous variables and the Mantel-Haenszel chi-square test or Fisher exact test, where appropriate, for discrete variables. Kaplan-Meier curves were used to compare the probability of clinical outcomes for the 1-year follow-up period by estrogen replacement status. The two study groups were compared with respect to clinical outcomes and composite end point at 1 year with use of the log rank test. The Fisher exact test was also used to evaluate angina class at 1 year for both groups. Because patients underwent 1-year follow-up at varying times, the angina class used as the 1-year follow-up value was obtained between 8 and 16 months after randomization. All tests of significance were two-tailed.

Multiple linear regression was used to assess the association

between late loss in minimal lumen diameter at follow-up and estrogen use after adjusting for multiple baseline clinical factors known to affect the rate of restenosis. The independent variables considered in this model included patient age, diabetes mellitus, current smoking, hypertension, high density lipoprotein level, unstable angina, vessel diameter, preprocedure minimal lumen diameter, presence of a lesion in the left anterior descending coronary artery, type of coronary intervention (angioplasty or atherectomy), and use or nonuse of estrogen replacement. To investigate whether the effect of estrogen use on restenosis differed between patients who underwent directional coronary atherectomy versus those who had coronary angioplasty, we included a formal test for interaction between coronary intervention and estrogen use. As this interaction term (coronary intervention  $\times$  estrogen use) was significant ( $p = 0.005$ ), we stratified our angiographic outcomes by the type of coronary intervention received. In addition, unadjusted univariable analyses of the independent variables considered in the multivariable model were used to evaluate correlates of late loss in minimal lumen diameter.

### Results

Of 243 women, 47 (19%) were using continuous unopposed estrogen or combined estrogen/progestin regimens before coronary intervention through the time of 1-year clinical follow-up. Thirty-eight (81%) of these women were taking unopposed conjugated equine estrogen (32 patients), estradiol ( $n = 4$ ) or estrone ( $n = 2$ ). Nine patients reported use of combined estrogen/progestin regimens. No woman reported initiating estrogen replacement therapy after coronary intervention or 6-month angiographic follow-up.

Characteristics of the patients. The baseline characteristics of the estrogen replacement users and nonusers are shown in Table 1. Women receiving estrogen replacement therapy were significantly younger, more likely to be smokers and more often had a positive family history of coronary artery disease. In addition, women using estrogen had significantly higher levels of high density lipoprotein and lower baseline rates of classes III and IV angina before coronary intervention. The two groups were similar with respect to factors associated with an increased risk of restenosis, including unstable angina and lesion location in the left anterior descending artery.

Immediate angiographic outcomes. The baseline angiographic data and immediate interventional outcomes in estrogen users and nonusers are displayed in Table 2. Percent stenosis before and after intervention was similar between estrogen users and nonusers. Women using estrogen replacement had smaller minimal lumen diameters before and after intervention ( $p = 0.05$  and  $0.055$ , respectively). However, the immediate gain for both estrogen users and nonusers varied little ( $0.93$  vs.  $0.97$  mm,  $p = 0.71$ ). Likewise, immediate success rate (defined as  $\leq 50\%$  stenosis by quantitative angiography) was high in both estrogen users and nonusers ( $89\%$  vs.  $89\%$ ,  $p = 1.0$ ).

Table 1. Baseline Clinical and Angiographic Characteristics

	Estrogen Users (n = 47)	Estrogen Nonusers (n = 196)	P Value
Age (yr)	60 (52, 67)	64 (57, 71)	0.003
Diabetes	25%	33%	0.31
Current smoker	30%	17%	0.054
Hypertension	62%	63%	0.89
Cholesterol (mg/dl)	233 (209, 261)	226 (199, 253)	0.34
High density lipoprotein (mg/dl)	54 (41, 60)	39 (32, 50)	0.002
Family history of CAD	61%	38%	0.005
Prior history of myocardial infarction	28%	41%	0.084
Comorbid disease*	38%	41%	0.70
CCS angina class III or IV	57%	73%	0.032
Unstable angina	81%	84%	0.64
Target vessel = LAD	54%	51%	0.71

\*Comorbid disease includes renal insufficiency (creatinine  $>1.5$  mg/dl), malignancy, chronic lung disease, rheumatologic disease, alcohol abuse, depression, physician-diagnosed obesity, gastrointestinal bleeding and cirrhosis. Data presented are median value (25th, 75th percentile) or percent of patient group. CAD = coronary artery disease; CCS = Canadian Cardiovascular Society; LAD = left anterior descending coronary artery.

Six-month follow-up. The angiographic follow-up data are displayed in Table 3 and Figure 1. The primary end point, the median late loss in minimal lumen diameter, was significantly lower in women using estrogen than in nonusers ( $-0.13$  vs.  $-0.46$  mm,  $p = 0.01$ ). In fact, univariable analysis of potential risk factors of restenosis revealed that estrogen use was the strongest predictor of late loss in minimal lumen diameter ( $F = 10.56$ ,  $p = 0.001$ ). In a multivariable analysis, the reduction in late loss remained significant after adjusting for potential risk factors for restenosis, including age, diabetes, current smoking, hypertension, high density lipoprotein level, unstable angina, vessel diameter, preprocedure minimal lumen diameter, presence of a lesion in the left anterior descending artery and type of coronary intervention (angioplasty or atherectomy). Multivariable regression analysis also found that estrogen use was the single most important predictor of subsequent late loss ( $F = 13.38$ ,  $p = 0.0006$ ). The only other

Table 2. Immediate Angiographic Outcomes

	Estrogen Users (n = 47)	Estrogen Nonusers (n = 196)	P Value
Preprocedure stenosis (%)	73 (63, 79)	72 (62, 78)	0.38
Postprocedure stenosis (%)	36 (29, 43)	35 (29, 45)	0.81
Preprocedure MLD (mm)	0.67 (0.48, 1.03)	0.82 (0.62, 1.06)	0.05
Postprocedure MLD (mm)	1.64 (1.42, 1.78)	1.75 (1.48, 2.06)	0.055
Acute gain (mm)*	0.93 (0.56, 1.19)	0.97 (0.62, 1.24)	0.71
Success rate†	89%	89%	1.0

\*Defined as postprocedure minimal lumen diameter minus preprocedure minimal lumen diameter. †Rate of reduction of stenosis to  $\leq 50\%$  as assessed by quantitative angiography. Data presented are median value (25th, 75th percentile) or percent of patient group. MLD = minimal lumen diameter.

Table 3. Angiographic Outcomes at Six-Month Follow-Up

	Estrogen Users (n = 41)	Estrogen Nonusers (n = 163)	p Value
Late loss (mm)*	-0.13 (-0.78, 0.12)	-0.46 (-0.80, -0.12)	0.01
Late loss index†	-0.136 (0.71, 0.17)	-0.52 (-0.92, -0.18)	0.005
Minimal lumen diameter (mm)	1.37 (0.85, 1.80)	1.24 (0.89, 1.68)	0.55
Rate of restenosis‡	41%	50%	0.26
Percent stenosis (%)§	48 (31, 60)	55 (38, 67)	0.18
Vessel caliber (mm)	2.5 (2.2, 3.0)	2.7 (2.4, 3.0)	0.03

\*Defined as 6-month follow-up minimal lumen diameter minus post-procedure minimal lumen diameter. †Late loss divided by acute gain in minimal lumen diameter. ‡>50% stenosis after an initially successful procedure. §Actual percent stenosis. Data presented are median value (25th, 75th percentile) or percent of patient group.

clinical factors found to significantly affect late loss were the intervention ( $F = 8.08$ ,  $p = 0.005$ ) and the interaction between the intervention and estrogen use ( $F = 7.22$ ,  $p = 0.008$ ).

Although the late loss index varied significantly between estrogen users and nonusers ( $-0.126$  vs.  $-0.522$ ,  $p = 0.005$ ), no significant differences existed when the other secondary angiographic end points were analyzed. However, similar trends toward improved outcomes were noted among estrogen users (Table 3). Despite having smaller lumen diameters before and after intervention, women using estrogen had a slightly larger median minimal lumen diameter at 6-month follow-up than did nonusers (1.37 vs. 1.24 mm,  $p = 0.55$ ). In addition, estrogen users tended to have a lower restenosis rate (>50% stenosis) than did nonusers when the dichotomous end point was used (41% vs. 50%,  $p = 0.26$ ).

As noted before, a predetermined analysis included investigating whether the effects of estrogen replacement therapy on angiographic restenosis varied between those who underwent atherectomy and those treated with conventional angioplasty. The testing for an interaction between coronary intervention and estrogen replacement use confirmed the existence

of this differential effect. Therefore Table 4 and Figures 2 and 3 display angiographic outcomes by coronary intervention and estrogen status. The median late loss in estrogen users undergoing atherectomy was 0.06 mm compared with  $-0.61$  mm in nonusers ( $p = 0.0006$ ). Likewise, the late loss index varied significantly between estrogen users and nonusers treated with atherectomy (0.06 vs.  $-0.63$ ,  $p = 0.002$ ). The median minimal lumen diameter in estrogen users undergoing atherectomy was larger than in nonusers (1.63 vs. 1.15 mm,  $p = 0.044$ ), and the dichotomous restenosis rate (>50% stenosis) in estrogen users undergoing atherectomy was 27% compared with 57% in nonusers ( $p = 0.038$ ). Little difference in minimal lumen diameter existed between estrogen users and nonusers who underwent angioplasty (1.26 vs. 1.32 mm,  $p = 0.34$ ). Similarly, the dichotomous restenosis rates were not significantly different between estrogen users and nonusers who received angioplasty.

**Clinical outcomes.** The clinical events and cumulative clinical outcome at 1-year follow-up are shown in Table 5. Six patients (3%) in the nonestrogen replacement group died within 365 days of coronary intervention. No deaths occurred in the estrogen replacement group. The rates of myocardial infarction, coronary artery bypass surgery and need for coronary intervention were slightly lower in estrogen users than in nonusers. The composite clinical end point (death, myocardial infarction, coronary artery bypass surgery and nonsurgical coronary intervention in the target artery) was similar between estrogen users and nonusers (15 events [31.9%] vs. 71 [35.5%],  $p = 0.75$ ). However, the number of patients who were classified as having class II, III or IV angina at follow-up demonstrated a trend toward less severe angina in estrogen users (5 patients [10.6%] vs. 31 [21.3%],  $p = 0.29$ ).

## Discussion

Restenosis remains a significant limitation for patients undergoing percutaneous coronary intervention, with 30% to

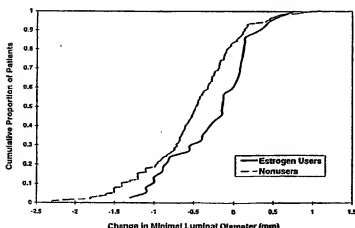


Figure 1. Cumulative frequency distribution of late loss in minimal lumen diameter of the target lesion in estrogen users and nonusers undergoing angioplasty and atherectomy.

Table 4. Angiographic Outcomes at Six-Month Follow-Up According to Intervention Assignment

	Atherectomy			Angioplasty		
	Estrogen Users (n = 18)	Estrogen Nonusers (n = 79)	p Value	Estrogen Users (n = 22)	Estrogen Nonusers (n = 84)	p Value
late loss (mm)*	0.06 (-0.15, 0.29)	-0.61 (-0.99, -0.23)	0.0006	-0.29 (-0.82, 0.09)	-0.39 (-0.66, -0.08)	0.77
late loss index†	0.06 (-0.20, 0.34)	-0.63 (-0.97, -0.21)	0.002	-0.40 (-0.81, 0.17)	-0.44 (-0.84, -0.08)	0.40
minimal lumen diameter (mm)	1.63 (0.83, 1.99)	1.15 (0.88, 1.49)	0.044	1.26 (0.88, 1.53)	1.32 (0.92, 1.72)	0.34
percent stenosis‡	27%	57%	0.038	48%	50%	0.69
percent stenosis (%)*§	31 (20, 59)	59 (38, 69)	0.01	51 (42, 66)	53 (44, 67)	0.78

\*Defined as postprocedure minimal lumen diameter minus 6-month follow-up minimal lumen diameter. †Late loss divided by acute gain in minimal lumen diameter. ‡Percent stenosis after an initially successful procedure. §Actual percent stenosis. Data presented are median value (25th, 75th percentile) or percent of patient group.

% of patients experiencing reocclusion within 6 months. 4. Unfortunately, clinical trials evaluating the efficacy of 30 pharmacologic agents have failed to demonstrate any significant success in modifying the rate of restenosis after balloon angioplasty (5-12). In contrast, our observational study revealed that the use of estrogen replacement therapy was associated with an overall reduction in late loss in target vessel lumen after percutaneous coronary intervention in postmenopausal women. This beneficial effect of estrogen replacement therapy appears to be significantly greater in women undergoing directional coronary atherectomy than in those receiving conventional angioplasty.

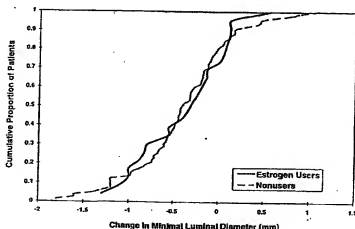
**Potential mechanism of action.** Several potential mechanisms may account for the ability of estrogen to attenuate restenosis. Multiple observational studies and a recent randomized trial (30) demonstrated that estrogen alone and in combination with a progestin in postmenopausal women lowers low density lipoprotein and increases high density lipoprotein levels. We also noted that women receiving estrogen replacement therapy had significantly greater high density lipoprotein levels. This favorable modulation of lipoprotein metabolism therefore may provide a partial explanation for the lower angiographic measures of restenosis after percutaneous coronary intervention. However, available data on the effects

of lovastatin (31), a more potent modifier of lipid profiles than estrogen, have failed to reveal any prevention of or reduction in restenosis. Likewise, significant differences between estrogen users and nonusers persisted after we adjusted for differences in high density lipoprotein levels. As a result, it seems improbable that the favorable lipoprotein effects of estrogen entirely account for the reduction in restenosis measures observed.

Estrogen has also been shown (16-19) to have direct effects on the vasomotion of normal and atherosclerotic arteries through endothelium-dependent and endothelium-independent mechanisms. Recent studies (20,21) have demonstrated that both short-term and long-term estrogen replacement therapy reverse the pathologic vasoconstriction observed in atherosclerotic vessels. These findings demonstrate that estrogen may promote vasodilation in diseased coronary arteries and potentially reduce pathologic vasoconstriction after percutaneous coronary intervention.

In addition, estrogen may prevent restenosis by altering cellular migration and neointimal proliferation after coronary intervention. After balloon-induced arterial injury, estrogen decreased platelet and neutrophil deposition at the site of injury (22). In vitro, physiologic levels of estrogen have been shown (23) to inhibit proliferation of vascular smooth muscle

Figure 2. Cumulative frequency distribution of late loss in minimal lumen diameter of the target lesion in estrogen users and nonusers undergoing angioplasty.



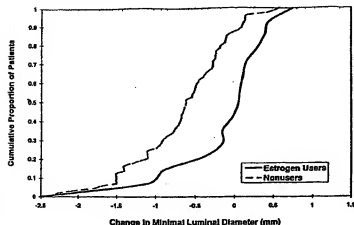


Figure 3. Cumulative frequency distribution of late loss in minimal lumen diameter of the target lesion in estrogen users and nonusers undergoing atherectomy.

from the coronary arteries of female pigs. Several studies have evaluated the ability of estrogen to alter vascular responses to injury in animal models. Although the results to date have been conflicting, the majority of studies (24–26) reported that estrogen significantly inhibited neointimal proliferation after arterial balloon injury. In contrast, a recent report by Geary and colleagues (27) concluded that estrogen did not inhibit balloon-induced proliferation of the intima in a nonhuman primate model.

**Device-specific response.** The present study was the first to clinically evaluate the effect of estrogen replacement therapy on restenosis in humans. We found an overall beneficial association between the use of estrogen replacement therapy and a reduction in late lumen loss after percutaneous coronary intervention. Although stratification by type of coronary intervention resulted in small subgroups ( $n = 97$  and  $107$  for atherectomy and angioplasty, respectively), we found that estrogen replacement therapy was associated with a significant reduction in restenosis in patients who underwent atherec-

tony, whereas the effects were equivocal after conventional angioplasty. In fact, there was no significant loss in median lumen diameter among the 18 patients using estrogen who received atherectomy as compared with a 36% loss in minimal lumen diameter in those not receiving estrogen therapy. Among those who received balloon angioplasty, estrogen users and nonusers had similar late angiographic results ( $-0.29$  vs.  $-0.39$  mm late loss in minimal lumen diameter,  $p = 0.77$ ).

These findings suggest that the effect of estrogen replacement therapy may be device specific. Such a device-specific response is also consistent with our current understanding of the pathophysiology of restenosis after coronary intervention. Although both neointimal hyperplasia and late recoil have been implicated in restenosis, the former may have a significantly greater role in restenosis after atherectomy than after conventional balloon angioplasty (32–34). Because previous *in vitro* and animal studies (23–26) have indicated that the presence of estrogen can reduce neointimal proliferation, our results demonstrating a greater impact of estrogen on restenosis after atherectomy may support this mechanism of action. However, further evaluation is necessary as our study is the first to investigate arterial responses to estrogen after atherectomy injury.

**Limitations.** This analysis was exploratory and several limitations need to be emphasized. Most important, the results are based on retrospective and nonrandomized data. Patients using estrogen replacement therapy differed from those not using estrogen. Although many differences in baseline characteristics between estrogen users and nonusers failed to reach statistical significance, the cumulative effect may account for the variance in angiographic outcomes. Estrogen users undergoing atherectomy were also significantly younger and had a greater family history of coronary artery disease than did nonusers. However, known potential confounding factors for restenosis were adjusted for in our primary end point analyses. Even after adjusting for diabetes, unstable angina and lesion location in the left anterior descending artery, the use of

Table 5. Clinical Outcomes at One-Year Follow-Up

	Estrogen Users ( $n = 47$ )	Estrogen Nonusers ( $n = 196$ )	<i>p</i> Value
Death	0	6 (3.0%)	0.24
Myocardial infarction	3 (6.4%)	14 (6.9%)	0.94
Coronary artery bypass surgery	3 (6.4%)	21 (10.3%)	0.44
Need for noncoronary coronary intervention in the target artery	12 (25.5%)	58 (28.6%)	0.69
CCS class II, III or IV*	5 (10.6%)	31 (15.3%)	0.29
Composite clinical end point†	15 (31.9%)	71 (35.5%)	0.75

\*Rates are based on proportion of patients with follow-up between 8 and 16 months after randomization. †The 1-year composite clinical end point was defined as death, myocardial infarction, coronary artery bypass surgery and noncoronary coronary intervention in the target artery. Data presented are number of patients (rates by Kaplan-Meier survival analysis). CCS = Canadian Cardiovascular Society.

estrogen replacement therapy remained the most significant factor predicting late loss.

A second limitation was our limited overall sample size, particularly when we stratified the analyses by type of coronary intervention used. This limited sample size increases the possibility of a chance or spurious finding. However, our primary angiographic end point was prospectively defined and ascertained and the reviewer did not know the patient's estrogen status. Despite this, confirmation of these subgroup analyses in larger clinical data bases, when available, is indicated.

The study's limited sample size also constrained our ability to demonstrate potentially significant differences in clinical outcomes between estrogen users and nonusers. Although estrogen users had slightly fewer deaths and myocardial infarctions and demonstrated a trend toward a lower rate of revascularization at 1-year follow-up, the study was not adequately powered to detect differences in clinical outcome. Recent preliminary evidence from Kim and colleagues (35) suggests that estrogen replacement therapy may improve long-term survival after percutaneous interventions in postmenopausal women.

**Conclusions.** This exploratory study suggests that estrogen replacement therapy in postmenopausal women may reduce restenosis after coronary intervention, particularly in patients receiving directional coronary atherectomy. Given the potential clinical importance of these findings, the use of estrogen replacement therapy as a means of preventing restenosis after coronary intervention deserves further evaluation.

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# The Biology of Restenosis

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Recent studies have allowed a better understanding of the biology of restenosis. Neointimal thickening—also referred to as neointimal hyperplasia—occurs in response to experimental arterial injury. This process involves different steps which include smooth muscle cell activation, proliferation, and migration, and the production of extracellular matrix. Neointimal thickening has been identified as one of the mechanisms of restenosis after balloon angioplasty in humans. The factors which control neointimal thickening include growth factors, hormonal factors, and mechanical factors. Delinquent reendothelialization has been shown to have a permissive impact on smooth muscle cell proliferation. In addition to neointimal thickening, arterial remodeling also plays a major role in restenosis. Studies performed on animals and in human subjects have established the potential for "constrictive remodeling" to reduce the vessel lumen after angioplasty. Restenosis thus appears as a multifactorial entity that may be addressed in the future by a combined mechanical and pharmacological approach.

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**P**ercutaneous transluminal coronary angioplasty (PTCA) has become a well-established technique for myocardial revascularization of patients with coronary artery disease. However, PTCA remains limited by restenosis that occurs in 30% to 60% of cases despite a successful procedure.<sup>1-4</sup> Assuming 500,000 PTCA procedures per year in the United States,<sup>5</sup> restenosis develops in more than 150,000 patients every year. Although some restenoses may be silent, most of these patients present with recurrent angina, and a significant proportion will need a new revascularization procedure. Decreasing the rate of restenosis would sharply lower the long-term cost of PTCA. In the United States, a reduction of the rate of restenosis from an hypothetical 33% to 25% might save as much as \$750 million annually.<sup>6</sup> Numerous agents have been used to prevent restenosis, and the results of more than 40

multicenter randomized clinical trials have now been published.<sup>6,7</sup> Despite intensive investigation in this area, no pharmacological therapy has yet been found to be useful in preventing restenosis.

The purpose of this article is to review the available information relevant to the mechanisms of restenosis. We first discuss the insights from animal experiments, and then focus on the mechanisms of restenosis in human beings.

## Evidence From Animal Experiments

### Quantitative and Qualitative Changes in Vessel Wall: The Healing Process After Arterial Injury

The healing response after arterial injury has been well characterized in various animal models of balloon denudation.<sup>1,8-10</sup> It begins immediately after the initial injury and may last for weeks or months. This "growth response" that leads to the development of a neointimal thickening, also known as neointimal hyperplasia, involves three key elements: smooth muscle cells (SMCs), endothelial cells, and the extracellular matrix.

### Smooth Muscle Cells

Immediately after arterial injury with a balloon catheter, multiple factors (discussion to follow) lead to the activation of SMCs. Early markers of SMC activation such as expression of nuclear oncogenes are detectable as soon as 30 minutes after injury.<sup>11,12</sup> This expression of nuclear oncogenes in the hours after angioplasty is associated with the early G1 events preceding DNA synthesis.

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sis in smooth muscle cells. Recently, the use of antisense oligonucleotides against nuclear oncogenes has been shown to inhibit neointimal thickening *in vivo* in various animal models,<sup>13,14</sup> suggesting that the inhibition of this early phase can significantly affect the cascade of events leading to neointimal hyperplasia a few weeks later.

Activation of SMCs is associated with a shift from a so-called contractile to synthetic phenotype<sup>15-17</sup> and leads to proliferation, migration, and synthesis of extracellular matrix. Proliferation of medial SMCs is evident 24 hours after experimental balloon injury and continues for at least 2 weeks.<sup>18</sup> At least 20% to 40% of medial SMCs are activated and enter the cell cycle between 24 hours and 3 days after balloon denudation<sup>18</sup>; these cells then migrate to the intima through breaks in the internal elastic membrane. Many of these neointimal cells continue to proliferate for several cycles, but nearly half of the migrating cells do not synthesize DNA.<sup>19</sup> Proliferation and migration should thus be considered as two distinct mechanisms leading to neointimal thickening; as will be discussed, some factors may affect SMC migration but have no effect on SMC proliferation, and vice versa.<sup>20</sup>

In animal models, the degree of intimal thickening is maximal after 3 months,<sup>21</sup> the additional volume that accumulates after 2 to 4 weeks reflects the adjunctive synthesis of extracellular matrix by synthetic SMCs (discussion to follow). After 2 to 3 months, SMCs return to a contractile phenotype and no further significant increase in intimal thickening occurs.<sup>22</sup>

#### Endothelial Cells

**Endothelial regeneration.** In the hours after experimental angioplasty, endothelial cells rapidly enter the replication cycle to restore endothelial continuity. Proliferation and migration can be initiated either by loss of contact inhibition, stretch, or growth factors secreted by endothelial cells, SMCs, and circulating cells.<sup>23,24</sup> Endothelial regeneration starts from the leading edge of the denuded area and from the ostia of collateral and/or branch arteries.<sup>24</sup> Re-endothelialization begins within the first 24 hours after the arterial denudation and ceases 6 or 10 weeks later, depending on animal species.<sup>25</sup> The rate and the extent of endothelial regrowth is related to animal

models and experimental conditions, but the most important factor seems to be the severity of the initial injury. Indeed, although complete re-endothelialization can be achieved after small denuding injuries,<sup>26,27</sup> endothelial cells have been found to be incapable of sustained regrowth after widespread denudation.<sup>23,28,29</sup> Even if complete re-endothelialization occurs, the regenerated endothelium often displays abnormal morphological characteristics. In contrast to normal endothelial cells, regenerating endothelial cells grow as a sheet with close cell-to-cell contacts, no longer aligned with blood flow, and are both polygonal in shape and irregular in size, with cytoplasm bulging toward the lumen.<sup>24,25,27,28,30</sup>

**Endothelial dysfunction.** The functional properties of regenerated endothelium have also been shown to be abnormal.<sup>27,29,31</sup> After vascular injury, endothelium-dependent relaxation to vasodilator agonists is depressed in arteries with regenerated endothelium at a time when the ability of underlying vascular SMCs to relax or contract is unchanged. The severity of the endothelial dysfunction appears to be dependent on the nature of the initial injury; in a rabbit iliac artery model of angioplasty, impaired endothelium-dependent relaxation was found 4 weeks after the procedure only if animals experienced severe injury.<sup>32</sup> Endothelial dysfunction may be related to a reduction in ability of endothelial cells to produce endothelium-derived relaxing factors.<sup>33</sup>

**Growth regulatory properties of endothelial cells.** Previous investigations have underscored the principle of cross-talk between endothelial cells and smooth muscle cells.<sup>34,35-38</sup> Neointimal thickness is closely related to the presence of a regenerated endothelium. Indeed, intimal areas that are rapidly covered by continuous endothelium are protected from the accumulation of intimal SMCs, whereas typical intimal hyperplasia occurs in areas where re-endothelialization is delayed.

Endothelium, in addition to its well-known role in regulating vessel tone and platelet aggregation, appears to modulate proliferative activity of the underlying SMCs. Recent *in vitro* studies have emphasized the possible role for nitric oxide (NO) in controlling SMC growth.<sup>37,39</sup> NO donors inhibit DNA synthesis in cultured SMCs, and similar effects achieved by administration of the analog 8-bromo-cyclic-guanosine monophosphate (8-bromo-cGMP) implicate the cGMP-pathway, physiologically activated by NO, in SMC

growth regulation. Endothelial cells may maintain SMC quiescence through the growth inhibitory effect of NO.<sup>34</sup> Dysfunctional, regenerating endothelium in which NO production is compromised may thus contribute to the development of a thickened intima because of reduced inhibition of platelet aggregation as well as SMC proliferation. It is intriguing to note that animal experiments have in fact documented retardation of endothelial cell coverage over damaged as opposed to normal media.<sup>35</sup> On the basis that nitric oxide synthase catalyzes the synthesis of NO after oxidation of terminal guanidino-nitrogen atoms of L-arginine,<sup>36,37</sup> several investigators have administered this precursor locally or systemically and confirmed *in vivo* the efficacy of NO in preventing intimal hyperplasia.<sup>38-42</sup>

Investigational experience with endothelial cell mitogens has yielded similar results. Asahara *et al* showed that a single local application of the endothelial cell-specific mitogen, vascular endothelial growth factor (VEGF), either as a recombinant protein<sup>43</sup> or as naked DNA,<sup>47</sup> is sufficient to facilitate endothelial repair after balloon injury.<sup>44</sup> In the gene transfer study, thrombosis and neointimal thickening were correspondingly attenuated to a statistically significant degree in arteries transfected with VEGF versus those transfected with a reporter gene; moreover, recovery of vasomotor reactivity was markedly accelerated. Similar results have been reported using basic fibroblast growth factor (bFGF).<sup>33,48</sup> The favorable effects of both VEGF and bFGF may be mediated by NO, as well as the fact that each of these mitogens has been shown to stimulate release of NO from endothelial cells.<sup>39,41</sup>

#### Extracellular Matrix

Experimental balloon denudation is followed by a marked increase in expression of the genes that code for extracellular matrix (ECM) proteins such as collagen and elastin in the arterial wall.<sup>23</sup> Similarly, the re-expression of embryonic forms of fibronectin occurs in the media and adventitia of rabbit arteries 24 to 48 hours after injury; 2 weeks after balloon denudation, when the neointima is formed, fibronectin mRNAs as well as the fibronectin protein accumulate in the luminal layers of the neointima.<sup>23</sup> Fibronectin, as well as other matrix constituents characterized by the presence of an Arg-Gly-Asp amino acid sequence such as osteo-

pontin<sup>44</sup> and vitronectin, are likely to facilitate SMC migration by interaction with certain integrins expressed by activated SMCs.<sup>33</sup> As pointed out by Schwartz *et al*,<sup>24</sup> cellular components constitute only about 11% of neointimal volume, and the remainder is ECM. Given the abundance of ECM in restenotic lesions, one alternative "anti-restenosis" strategy would be to directly reduce the matrix volume surrounding each cell; whether a substantial reduction in neointimal volume can be achieved without inhibition of SMC migration and/or proliferation remains to be seen.

#### Potential Regulators of the Healing Process

**Platelets and the thrombotic process.** Immediately after experimental balloon injury, endothelial denudation induces platelet adhesion and aggregation resulting in release of the constituents of their alpha granules within a few minutes.<sup>2,27</sup> Numerous mitogenic substances, including platelet derived growth factor (PDGF), are thus released at the site of injury and may be involved in the process of SMC activation.<sup>28</sup> Experiments performed in thrombocytopenic animals have shown the fundamental role of platelets in determining the extent of neointimal thickening after arterial injury.<sup>29</sup> Experiments performed in a canine model of endothelial injury have shown that the intensity of cyclic flow variations related to platelet accumulation constitute a major determinant of neointimal thickening.<sup>30</sup> Recent studies have shown that thrombocytopenia inhibits migration of activated SMCs from the media to the intima but has no effect on the initial cycle of cell proliferation.<sup>31</sup> Coagulation proteins such as thrombin may also be implicated in the response of SMCs.<sup>45</sup> Thrombin has mitogenic properties for SMCs<sup>46</sup> and has been shown to induce multiple growth-related signals in SMCs including the expression of the *c-fos* proto-oncogene.<sup>49</sup> Finally, the volume of thrombus at the PTCA site may also play a role in the subsequent restenotic process; Schwartz *et al*,<sup>24</sup> based on observations made in the porcine coronary injury model, proposed that the volume of intracoronary thrombus resulting from PTCA may serve as a scaffold that determines the subsequent volume of neointima.

**Growth factors.** Growth factors released at the site of injury play a major role in the response of

SMCs to balloon injury. Platelets are an important source of PDGF, but endothelial cells, macrophages, and SMCs may themselves secrete PDGF after arterial injury.<sup>15,26-28</sup> PDGF may be critical for SMC migration from media to intima, whereas its absence does not limit SMC proliferation.<sup>27,28</sup> Using an antibody to PDGF, Ferns et al were able to reduce neointimal SMC accumulation after experimental angioplasty without affecting mitogenic activity.<sup>29</sup>

Basic fibroblast growth factor (bFGF), an *in vivo* angiogenic factor<sup>30</sup> that is mitogenic for SMCs as well as endothelial cells through specific receptors,<sup>31,32</sup> may also play a role in restenosis. SMCs within the tunica media, when damaged by an oversized balloon, may release bFGF because of stretch or crush injury; the liberated bFGF can then mediate the initial wave of cell division within this layer of the blood vessel. Infusion of an antibody that neutralizes bFGF reduces the first cycle of SMC replication by up to 80% in the arterial media after balloon denudation but has no effect on the resulting neointimal thickening.<sup>33</sup> Alternatively, bFGF may potentially limit the restenotic process via its effect on endothelium. Recent studies have documented that exogenous administration of bFGF may accelerate endothelial regrowth after balloon denudation.<sup>33,34</sup> As stated, similar effects have been observed with VEGF.<sup>34,35</sup>

Additional growth factors that have been implicated in the development of restenosis include transforming growth factor  $\beta$  (TGF $\beta$ ) and insulin growth factor type 1 (IGF-1). TGF $\beta$  mRNA is increased in SMCs after arterial wall injury and reaches a maximum before the phase of extracellular matrix synthesis.<sup>32,33</sup> TGF $\beta$  is known to modulate fibronectin expression<sup>36</sup> and may be important in the control of extracellular matrix synthesis.<sup>32,33</sup> TGF $\beta$  is produced by SMCs, platelets, and endothelial cells.<sup>36,37</sup> The main source of IGF-1 is SMCs, and its mRNA expression undergoes a 10-fold increase in the weeks after balloon denudation.<sup>38</sup>

**Hormonal factors.** Work by Dzau et al has suggested a role for the renin-angiotensin-aldosterone axis in the pathogenesis of restenosis.<sup>39,40</sup> Angiotensin II may modulate SMC growth *in vitro*.<sup>41,42</sup> *In vivo* studies have shown that angiotensin II infusion in rats is followed by marked SMC proliferation in the intima.<sup>43</sup> Powell et al have reported that inhibitors of angiotensin

converting enzyme (ACE) suppress myointimal proliferation after vascular injury.<sup>44</sup> The actions of ACE inhibitors are probably not solely related to an effect on angiotensin II levels, but may in part be caused by an effect on bradykinin metabolism<sup>45,46</sup> or by effects mediated by aldosterone.<sup>47</sup> Serotonin and other vasoactive hormones (catecholamines, vasopressin) released at the angioplasty site are also able to induce SMC proliferation.<sup>48-51</sup> Recently considerable interest has been focused on endothelin, a potent vasoconstrictor peptide produced by vascular endothelial cells.<sup>52</sup> Endothelin binds specifically to human SMCs in culture and can induce nuclear oncogene expression.<sup>53,54</sup> Endothelin has been shown to have mitogenic activity for rat aortic SMCs,<sup>55</sup> and endothelin antagonists reduce neointimal thickening after vascular injury *in vivo*.<sup>54,57</sup>

**Mechanical factors.** Mechanical factors may also play a role in determining the magnitude of the restenotic lesion generated in experimental models. Large areas of endothelial denudation without trauma to the media lead to mild neointimal thickening despite late endothelial regeneration<sup>58</sup>; in contrast, focal endothelial denudation with substantial medial trauma, including rupture of the internal elastic lamina, is associated with marked neointimal proliferation, despite prompt endothelial regeneration.<sup>59</sup> These results suggest that endothelial denudation alone is not sufficient to produce major neointimal thickening and that direct stimulation of SMCs is also an important factor. Direct injury of SMCs may induce a greater neointimal response by (1) increasing the local release of growth factors from necrotic SMCs, and (2) activation of intracellular pathways, leading to proliferation of residual viable SMCs.<sup>58,60</sup>

In addition to acute mechanical factors, chronic mechanical factors may also play a role. Blood flow in the injured vessel may be a determinant of subsequent restenosis; experimental studies by Koller et al have shown greater neointimal hyperplasia when blood flow is reduced after angioplasty.<sup>61,62</sup>

#### Changes in Lumen Size: The Concept of Vascular Remodeling

Arterial remodeling is now a well-recognized feature of *de novo* atherosclerosis. Glagov et al<sup>63</sup> observed that human coronary arteries undergo adaptive enlargement in response to progressive

plaque expansion and maintain the lumen area until the plaque occupies 40% of the area circumscribed by the internal elastic lamina. This so-called compensatory enlargement may thus obviate the effect of plaque development on lumen narrowing.

There is increasing experimental evidence that neointimal hyperplasia is not the sole mechanism leading to lumen renarrowing after angioplasty, and that arterial remodeling also plays a major role in this process.<sup>105-107</sup> In the hypercholesterolemic rabbit model, Kakuta et al.<sup>108</sup> showed that compensatory enlargement of the vessel (increase in internal elastic lamina area) occurs in the weeks after experimental angioplasty; this process appeared to compensate for nearly 60% of neointimal thickening in response to balloon injury and prevent lumen narrowing. Surprisingly, restenosis was not related to neointimal thickening, but to a lack of compensatory enlargement and/or some degree of vessel constriction. Vascular remodeling thus acts to offset the effect of neointimal formation on chronic luminal narrowing, and differences in vascular remodeling appear to constitute a key element leading to restenosis in this model. Other reports by Post et al.<sup>109</sup> and Lafont et al.<sup>107</sup> have confirmed the role of vascular remodeling in other models of restenosis.

The precise mechanisms responsible for arterial remodeling after balloon injury are unknown. Proposed mechanisms for compensatory enlargement in *de novo* arteriosclerosis include increased flow with concomitant increase in shear stress and modulation of the ECM support structure with protrusion of the lesion outside the original contour of the artery.<sup>104</sup> Post-balloon angioplasty, compensatory enlargement in response to neointimal growth may occur as a result of high flow velocity or as a consequence of ECM degradation.<sup>106</sup> Chronic constriction, on the other hand, may be related to collagen deposition and reorganization.<sup>108</sup>

Several studies have also suggested a potential role for the endothelium in modifying the remodeling process. Previous studies have shown that adaptive enlargement, i.e., increase in the lumen area of the vessel, occurs in response to a long-term increase in flow velocity<sup>110</sup>; conversely, a structural decrease in the lumen diameter is associated with a long-term decrease in blood flow. It appears that a functional endothelium is

essential for the compensatory arterial response to long-term changes in luminal flow rates.<sup>110</sup> Thus the absence of endothelium or the presence of a regenerated dysfunctional endothelium after arterial injury may impair the shear stress-induced remodeling response and may be an important factor of restenosis.<sup>111</sup>

### Human Restenosis

An important issue is to determine whether evidence for certain pathophysiological mechanisms of restenosis shown in experimental models can be supported by clinical observations in human subjects. Although the arterial wall response after coronary angioplasty in human beings has been exhaustively detailed by angiographic end-point studies, serial evaluation with or without tissue specimens has been less well documented than responses in experimental animal models. Nevertheless, contemporary diagnostic tools of invasive cardiology such as directional atherectomy, intravascular ultrasound (IVUS), and angioscopy have allowed a better description and understanding of this process in human beings.

#### Pathology of Restenosis in Human Beings

##### Light-Microscopic Findings

Light-microscopic features of atherectomy specimens retrieved from coronary arteries of patients undergoing percutaneous revascularization<sup>112,113</sup> have confirmed observations made in smaller numbers of patients at necropsy.<sup>114,115</sup> Primary and restenosis lesions can often be distinguished from each other on the basis of certain light-microscopic findings.<sup>116</sup> Primary lesions appear typically hypocellular, consisting predominantly of well-organized collagen and ground substance. In contrast, restenosis lesions typically include a focus of hypercellularity; cells within these foci have phenotypic characteristics of proliferative SMCs, and the extracellular matrix surrounding these cells has a less compact appearance than does the extracellular matrix of primary plaque. However, it must be acknowledged that this hypercellular lesion is not altogether specific for restenosis: in the CAVEAT investigation, a similar lesion was observed in 7% of primary specimens.<sup>118</sup>

### Evidence of Smooth Muscle Cell Proliferation

Immunohistochemical analyses have been performed to determine the degree of cell proliferation in specimens retrieved with the use of directional atherectomy.<sup>117</sup> Pickering et al have analyzed the expression of the proliferating cell nuclear antigen (PCNA) and have reported that the proliferative index (percent of PCNA-positive cells) was higher in restenotic than in primary lesions. Proliferating cells were detected by *in situ* hybridization and immunohistochemistry in 15% to 20% of cells of specimens retrieved from restenosis lesions. These findings thus indicate that, among patients referred for percutaneous revascularization, many have a detectable proliferative component. More recently, the greater SMC proliferative activity documented for restenosis lesions has been shown to be associated with evidence of programmed cell death.<sup>118</sup>

### The Extracellular Matrix

Although vascular SMC proliferation and the resulting hypercellular nature of the fibroproliferative tissue have been the focus of most studies of restenosis, it is the ECM that in fact accounts for the bulk volume of these lesions. Together with activated SMCs, ECM having a distinctively lighter hue on histochemical staining comprises the classic restenosis focus.<sup>84</sup> Various collagen subtypes and proteoglycans have been shown to comprise the ECM of atherectomy specimens.<sup>119</sup> The contribution of two proteoglycans in particular, biglycan and decorin, known to be differentially modulated during ECM elaboration and differentially regulated by TGF- $\beta_1$ , have been found to be differentially distributed in primary versus restenotic lesions.<sup>119</sup> Extracellular deposits of biglycan were found to be characteristic of the loose, rich extracellular matrix typical of restenosis, whereas staining for decorin was limited to weak staining intensity in the more compact transition zone between loose ECM and dense connective tissue typical of advanced lesions composed of hypocellular fibrous plaque.

More recently, the glycosaminoglycan hyaluronan has been shown to be a characteristic constituent of the loose myxoid ECM of human restenotic arteries.<sup>120</sup> The presence of hyaluronan may be a marker for an initial phase of the ECM remodeling that occurs during the development of a fibroproliferative lesion and could facilitate bio-

logical processes such as cell migration. Furthermore, animal studies performed in our laboratory suggest that hyaluronan deposits are most robust early in neointimal development. The end-stage lesion typical of *de novo* or primary atherosclerosis thus contains limited deposits of hyaluronan. Restenotic lesions contain more. If maturation of the restenotic lesion is similar to that in the initial lesion, then even the increased deposits seen at the time of atherectomy may be less than was present early on, after revascularization. Gradual replacement of this bulky, heavily hydrated molecule by collagen with resulting retraction of the mature scar (including wall) could represent a biochemical correlate of vascular remodeling.

### Insights From IVUS Studies

As in experimental models, neointimal hyperplasia appears not to be the sole mechanism of restenosis in human beings. IVUS studies suggest that vascular remodeling also occurs after angioplasty in human beings. Studies by Mintz et al<sup>121</sup> have been interpreted to show that most of the late lumen loss after balloon angioplasty is due to arterial remodeling, rather than intimal thickening.

### Insights From Angioscopic Studies

We have used serial coronary angioscopy to describe the morphological changes occurring in the months following percutaneous transluminal coronary angioplasty of unstable plaques.<sup>122</sup> Angioscopy was performed immediately before PTCA, immediately after PTCA, and at 6-month follow-up. Although the angioscopic appearance of the lesion at the time of angioplasty was typically that of a complex yellow plaque with superimposed thrombus, the angioscopic appearance at follow-up was almost invariably that of a stable smooth white plaque without thrombus. These differences reflect the magnitude of the changes occurring at the lesion site during the follow-up period. In addition to disappearance of dissection and thrombi, the smooth concentric white aspect of the plaque is likely to be a consequence of neointimal hyperplasia leading to a remodeling of the inner part of the vessel. Whether the PTCA site is completely re-endothelialized at follow-up cannot be determined using angioscopy.

### Conclusion

Restenosis is clearly a multifactorial entity. SMC proliferation, elaboration of ECM, thrombosis, and vascular remodeling all contribute to its pathogenesis. The contribution of these individual elements does not appear to be consistently proportional from one patient to another, or even one lesion to another in the same patient. In the absence of a single therapy that effectively addresses all of these pathogenetic elements, the ability to routinely preempt restenosis will likely require accurate clinical identification and understanding of the principle factor governing the biology of a given restenotic lesion.

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# United States Patent [19]

Pitha

[11] Patent Number: 4,727,064

[45] Date of Patent: Feb. 23, 1988

## [54] PHARMACEUTICAL PREPARATIONS CONTAINING CYCLODEXTRIN DERIVATIVES

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[73] Assignee: The United States of America as represented by the Department of Health and Human Services, Washington, D.C.

[21] Appl. No.: 738,749

[22] Filed: May 29, 1985

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[63] Continuation-in-part of Ser. No. 603,839, Apr. 25, 1984, Pat. No. 4,596,795.

[51] Int. Cl.<sup>1</sup> ..... A61K 31/70; C08B 37/16

[52] U.S. Cl. .... 514/58; 106/210;

536/103; 514/965; 514/971

[58] Field of Search ..... 536/103; 106/210;

514/58, 971, 965

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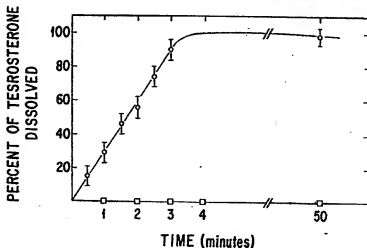
Primary Examiner—Ronald W. Griffin  
Attorney, Agent, or Firm—John S. Roberts, Jr.

### [57]

### ABSTRACT

The invention comprises pharmaceutical preparations consisting generally of a drug with a substantially low water solubility and an amorphous, water-soluble cyclodextrin-based mixtures. In these preparations a stable amorphous state can be achieved. This improves the dissolution properties of the drug and hence its absorption by the body. The required cyclodextrin-based mixtures were prepared from  $\alpha$ -,  $\beta$ -, or  $\gamma$ -cyclodextrin which were rendered amorphous through non-selective alkylation. The alkylation agents suitable for that purposes are exemplified by propylene oxide, glycidol, isooctacetamide, chloroacetate, or 2-diethylaminoethylchloride; their reactions with cyclodextrins were performed in a manner to yield mixtures containing many components, a circumstance which effectively prevents crystallization processes within the above pharmaceutical preparation.

28 Claims, 2 Drawing Figures



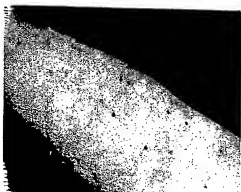


FIG. 1B

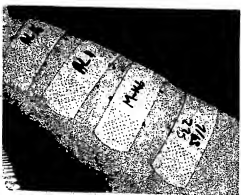


FIG. 1A

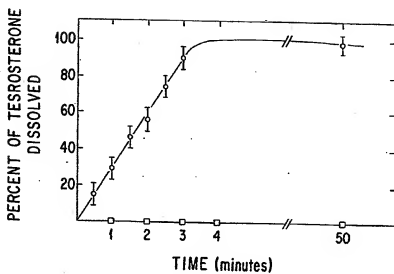


FIG. 2

# PHARMACEUTICAL PREPARATIONS CONTAINING CYCLODEXTRIN DERIVATIVES

This application is a continuation-in-part of pending Ser. No. 603,839 filed April 25, 1984, now U.S. Pat. No. 4,596,795.

This invention comprises pharmaceutical preparations consisting generally of a drug with a substantially low water solubility and an amorphous, water-soluble cyclodextrin-based mixtures. In these preparations a stable amorphous state can be achieved and that improves the dissolution properties of the drug and hence its absorption by the body. The required cyclodextrin-based mixtures were prepared from  $\alpha$ ,  $\beta$ , or  $\gamma$ -cyclodextrin which were rendered amorphous through non-selective alkylation. The alkylation agents suitable for that purpose are exemplified by propylene oxide, glycidol, iodoacetamide, chloroacetate, or 2-diethylaminoethylchloride; their reactions with cyclodextrins were performed in a manner to yield mixtures containing many components, a circumstance which effectively prevents crystallization processes within the above pharmaceutical preparation.

This method of improvement of pharmaceutical preparation comprises the addition of crystalline drugs with substantially low solubility to compounds which have the following characteristics: (a) are water-soluble cyclodextrins, (b) have the ability to form inclusion complexes with the drugs in question, (c) are intrinsically amorphous and substantially decrease the tendency of the drug to crystallize. The above addition results in an improved solubility of the drug composition in question and more efficient absorption of the drug by the body.

The parent cyclodextrins are converted by alkylation with, e.g., epoxides or organic halides into mixtures of substituted cyclodextrins. These mixtures of cyclodextrin derivatives, after separation from contaminating products of self-condensation of epoxides or halides, are used as such, i.e., without separation into the individual cyclodextrin derivatives. Use of such water-soluble mixtures of cyclodextrin derivatives in pharmaceutical preparations enables stabilization of the amorphous state and leads to better absorption of drugs by a body.

## BACKGROUND OF THE INVENTION

This invention is directed to the method of conversion of drug compositions which themselves are crystalline and of low water-solubility into intrinsically amorphous complexes which have improved pharmaceutical properties. This conversion is achieved by inclusion of the above drug compositions into water-soluble, multi-component mixtures of cyclodextrin derivatives. More particularly, the invention is related to compounding application Ser. No. 603,839 which concerned cyclodextrins and sex hormones.

## MATERIAL INFORMATION DISCLOSURE

Cyclodextrins are cyclic oligomers of glucose; these compounds form inclusion complexes with any drug whose molecule can fit into the lipophile-seeking cavities of the cyclodextrin molecule. Cyclodextrins are crystalline and so are their complexes with drugs, and these complexes have somewhat improved water solubilities compared to the drugs themselves. The latter

improvements are the subject of reviews and of numerous patents (J. Szejtli, "Cyclodextrins and Their Inclusion Complexes," Akademiai Kiado, Budapest, 1982, pp. 204-232; J. Pitha, L. Sente, and J. Szejtli, "Molecular Encapsulation of Drugs by Cyclodextrins and Congeners," in *Controlled Drug Delivery*, S. D. Bruck, ed., CRC Press Inc., Boca Raton, FL, 1983, pp. 125-148).

Chemical modifications of cyclodextrins have been widely performed; with a few exceptions nevertheless the aim was preparation of another individual crystalline compound (M. L. Bender and M. Komiyama, "Cyclodextrin Chemistry," Springer-Verlag, Berlin, 1978, pp. 29-32). Condensation reactions of cyclodextrins with various epoxides or organic halides yielding compounds which are principally similar to those presently used have been known: (1) electroneutral, soluble cyclodextrin derivatives were described by Parmerter et al., U.S. Pat. No. 3,453,259, July 1969; Gramera et al., U.S. Pat. No. 3,459,731, Aug. 1969; (2) cationic, soluble cyclodextrin derivatives were described by Parmerter et al., U.S. Pat. No. 3,453,257, July 1969; and (3) insoluble, crosslinked cyclodextrins were described by Solms, U.S. Pat. No. 3,420,788, Jan. 1969. The above mixtures have not been used to obtain amorphous and water-soluble pharmaceutical preparations. Water-insoluble (crosslinked) polymers of  $\beta$ -cyclodextrins were previously tested as tabletting additives (E. Fenyvesi, et al., *Chem. Pharm. Bull.*, 32:665, 1984; E. Fenyvesi, et al., *Chem. Pharm. Bull.*, 32:670, 1984). Furthermore, cross-linked  $\alpha$ -cyclodextrin was previously used in the preparation of sustained-release formulation of penicillin (Japanese Kokai Tokkyo Koho Patent No. 82-130914).

## SUMMARY OF THE INVENTION

Dissolution properties of drugs may be improved by their conversion to an amorphous state or by complexation with cyclodextrins. The present invention combines these two improvements: described is the preparation and use of mixtures of cyclodextrin derivatives which are intrinsically amorphous, water-soluble, and capable of forming inclusion complexes with drugs. These cyclodextrin mixtures effectively solubilize lipophilic drugs into aqueous media. The solutions of these cyclodextrin mixtures are non-irritating topically and in difference to the solutions of cyclodextrins themselves do not support microbial growth. Thus, these compounds are well suited as additives/solubilizers in topical preparations. The solutions of the above cyclodextrin mixtures furthermore have very low toxicity, both systemic and local, when applied parenterally. Thus, they are well suited as additives for parenteral preparations. Furthermore, when solutions of these cyclodextrin mixtures are saturated with drugs and then evaporated or freeze-dried, solids are obtained which dissolve easily and completely and are stable for an extended period of time. These solids can be either directly tableted to yield products well suited for oral or buccal administration or processed into suppositories.

The cyclodextrin additives may be utilized in weight percent of usually about 40-60% of the drug solution composition and may be utilized from about 5-95% of the drug solution composition. Actual working models of the cyclodextrin derivatives are set out in Table I, which shows some of the preferred as 40-50% of the drug solution composition.

TABLE I

Drug	Solubilization of Various Drugs	
	Solubility in water (mg/ml)	Solubility in solubilizer solution Solubilizer <sup>a</sup> (conc. in water) Solubility (mg/ml)
acetamidophen	11	hydroxypropyl- $\beta$ -cyclodextrin (50%) 67.0
apomorphine	20	hydroxypropyl- $\beta$ -cyclodextrin (50%) 116.0
butylated hydroxytoluene	insoluble	hydroxypropyl- $\alpha$ -cyclodextrin (40%) 0.3
butylated hydroxytoluene	insoluble	hydroxypropyl- $\beta$ -cyclodextrin (50%) 3.0
butylated hydroxytoluene	insoluble	hydroxypropyl- $\gamma$ -cyclodextrin (40%) 0.2
chlorthalidone	<0.12	hydroxypropyl- $\beta$ -cyclodextrin (50%) 10.5
cholecalciferol	<0.23	hydroxypropyl- $\beta$ -cyclodextrin (50%) 10.0
desamethasone	0.1	hydroxypropyl- $\beta$ -cyclodextrin (50%) 24.0
dicumaryl	<0.15	hydroxypropyl- $\beta$ -cyclodextrin (50%) 1.3
digoxin	0.07 <sup>2</sup>	hydroxypropyl- $\beta$ -cyclodextrin (50%) 45.0
diphenhydramine	0.03 <sup>2</sup>	hydroxypropyl- $\beta$ -cyclodextrin (50%) 1.7
estradiol	<1.6	hydroxypropyl- $\beta$ -cyclodextrin (40%) 28.0
estradiol	<1.6	carboxymethyl- $\beta$ -cyclodextrin (50%) 25.0
estradiol	<1.6	carboxymethyl- $\beta$ -cyclodextrin (50%) 10.0
estril	<1.3	hydroxypropyl- $\beta$ -cyclodextrin (50%) 41.0
ethylethyl-3-methyl ether	<1.5	hydroxypropyl- $\beta$ -cyclodextrin (50%) 27.0
chloroform	<0.2	hydroxypropyl- $\beta$ -cyclodextrin (50%) 0.5
furosemide	0.07 <sup>2</sup>	hydroxypropyl- $\beta$ -cyclodextrin (50%) 1.7
hydroflumethiazide	0.3	hydroxypropyl- $\beta$ -cyclodextrin (50%) 9.3
indomethacin	0.02 <sup>2</sup>	hydroxypropyl- $\beta$ -cyclodextrin (50%) 4.2
iproniazid phosphate	30	hydroxypropyl- $\beta$ -cyclodextrin (50%) 55.0
17-methyltestosterone	<0.17	hydroxypropyl- $\beta$ -cyclodextrin (50%) 39.0
nitroglycerin	1.25	hydroxypropyl- $\alpha$ -cyclodextrin (40%) 8.7
nitroglycerin	1.25	hydroxypropyl- $\beta$ -cyclodextrin (40%) 10.4
nitroglycerin	1.25	hydroxypropyl- $\gamma$ -cyclodextrin (40%) 9.8
norethindrone	2.5	hydroxypropyl- $\beta$ -cyclodextrin (50%) 6.8
ouabain	13	hydroxypropyl- $\beta$ -cyclodextrin (50%) 80.0
oxprenolol	12	hydroxypropyl- $\beta$ -cyclodextrin (50%) 238.0
progesterone	0.01 <sup>3</sup>	hydroxypropyl- $\beta$ -cyclodextrin (40%) 34.0
retinal	<0.07	hydroxypropyl- $\beta$ -cyclodextrin (40%) 2.6
retinoic acid, all trans	<0.07	hydroxypropyl- $\beta$ -cyclodextrin (40%) 0.8
retinoic acid, choline salt of all trans		hydroxypropyl- $\beta$ -cyclodextrin (40%) 18.4
retinoic acid, ethanolamine salt of all trans		hydroxypropyl- $\beta$ -cyclodextrin (40%) and ethanolamine (1%) 28.4
retinoic acid, sodium salt of all trans		hydroxypropyl- $\beta$ -cyclodextrin (40%) 1.6
retinol	<0.10	hydroxypropyl- $\beta$ -cyclodextrin (40%) 5.5
spirinolactone	0.01 <sup>2</sup>	hydroxypropyl- $\beta$ -cyclodextrin (40%) 42.0
sulpiride	<0.21	hydroxypropyl- $\beta$ -cyclodextrin (50%) 10.0
testosterone	0.02 <sup>4</sup>	hydroxypropyl- $\beta$ -cyclodextrin (40%) 38.0
testosterone	0.02 <sup>4</sup>	carboxymethyl- $\beta$ -cyclodextrin (50%) 24.0
testosterone	0.02 <sup>4</sup>	carboxymethyl- $\beta$ -cyclodextrin (50%) 30.0
thiophylline	8.3	hydroxypropyl- $\beta$ -cyclodextrin (50%) 11.0
acyclovir	insoluble	hydroxypropyl- $\beta$ -cyclodextrin (50%) 2.5
c/ciside hydrochloride		hydroxypropyl- $\beta$ -cyclodextrin (50%) 33.0
testosterone	0.02 <sup>4</sup>	dihydroxypropyl- $\beta$ -cyclodextrin (40%) 25.0

<sup>a</sup>Degrees of substitution of solubilizers used: hydroxypropyl- $\beta$ -cyclodextrin and homologs, 6-7; diethylaminoethyl- $\beta$ -cyclodextrin, 1.5; carboxymethyl- $\beta$ -cyclodextrin, 4; carboxymethyl- $\beta$ -cyclodextrin, 3.

<sup>2</sup>Data from K. Uekama, *Pharmaceutical International*, 41-43 (1975).

<sup>3</sup>Data from J. Brotherton, *Sea Hormone Pharmacology*, Academic Press, New York (1976), p. 36.

### DESCRIPTION OF THE FIGURES

FIGS. 1(A and B) shows the activity of derivatives of cyclodextrins on the forearm with a band-aid patch, showing lack of topical irritation. On the left is a photograph of the forearm with band-aid patches wetted with isotonic solutions in water of AL16 (carboxamidomethyl- $\beta$ -cyclodextrin), AL11 (carboxymethyl- $\beta$ -cyclodextrin), M146 (hydroxypropyl- $\beta$ -cyclodextrin), and SzA59/2 (diethylaminoethyl- $\beta$ -cyclodextrin). On the right is the forearm after band-aids were removed after 18 hours of exposure.

FIG. 2 shows the dissolution of testosterone from a tablet made from testosterone/hydroxypropyl- $\beta$ -cyclodextrin complex (—) or from a tablet made from testosterone-microcrystalline cellulose (—□—).

### PREPARATION OF CYCLODEXTRIN MIXTURES

Suitability of cyclodextrins as pharmaceutical additives/solubilizers can be considerably improved if these are converted to mixtures of derivatives which are

highly soluble and retain the capacity to form inclusion complexes with drugs. Such mixtures may be prepared by alkylation reactions which lack chemoselectivity and stereoselectivity and introduce hydrophilic substituents. In the examples are shown such alkylations.

Tables 1-3 below show solubility or dissolution of various compounds under option of cyclodextrin derivatives.

Systemic and Local Toxicity of Cyclodextrin Derivatives

The lack of topical irritation of the above cyclodextrin derivatives was tested by wetting a band-aid with their isotonic solutions and by application of that band-aid to the forearm for about 18 hours. The representative results can be seen in FIG. 1.

The lack of parenteral toxicity of the above cyclodextrin derivatives was tested by intraperitoneal injection into mice; the following results were obtained.

(a) Two different preparations of hydroxypropyl- $\beta$ -cyclodextrin were tested. The first preparation (degree

of substitution 6), in doses of 10 g/kg, was without lethal effects (4 animals). The second preparation (degree of substitution 8), at 6.3 g/kg, resulted in 2 deaths (after 4 days) and 1 surviving animal; at 3.2 g/kg all 4 animals survived.

(b) Diethylaminoethyl- $\beta$ -cyclodextrin at 4.7 g/kg—1 death (after 4 hrs) and 2 surviving animals; at 2.4 g/kg all 3 animals survived.

(c) Carboxymethyl- $\beta$ -cyclodextrin at 3.8 g/kg—no deaths, 3 surviving animals.

(d) Carboxamidomethyl- $\beta$ -cyclodextrin at 6.9 g/kg—2 deaths, 1 surviving animal; at 3.5 g/kg all 4 animals survived.

The lack of oral toxicity of hydroxypropyl- $\beta$ -cyclodextrin in mice was documented in patent application Ser No. 603,839 filed April 25, 1984, by Pitha.

**Dissolution Effects of Cyclodextrin Derivatives**  
Effectiveness of cyclodextrin derivatives in assisting dissolution of drugs is satisfactory if (a) a substantial part of the drug molecule can be fitted into the hydrophobic cavity of the cyclodextrin molecule, and (b) the same part of the drug molecule is hydrophobic. Since these principles were established previously (see Material Information Disclosure section), the present efforts have been directed to dividing drugs into categories according to their steric bulk and documenting the usefulness of the present method for a few representatives of every category.

**Improved Absorption of Drugs from the Preparations Described Above**

The principal improvement of absorption of a drug by a body accompanying the conversion of a drug from a crystalline state into an amorphous state is generally recognized.

TABLE 2

	Solubilities of Steroids (mg/ml) in 50% Aqueous Solutions of Hydroxypropyl- $\beta$ -cyclodextrin Samples With Different Degrees of Substitution (d.s.)			
	d.s. 4.7	d.s. 5.7	d.s. 7.0	d.s. 14
Estradiol	30	35	26	18
Progesterone	30	44	35	23
Testosterone	56	62	40	23

TABLE 3

	Solubilities of Steroids (mg/ml) in 50% Aqueous Solutions of Substituted Cyclodextrins		
	Carboxamidomethyl- $\beta$ -cyclodextrin	Diethylaminoethyl- $\beta$ -cyclodextrin	Carboxymethyl- $\beta$ -cyclodextrin
Estradiol	28	8	14
Progesterone	38	18	14
Testosterone	24	9	24

## EXAMPLE 1

**Preparation of hydroxypropyl- $\beta$ -cyclodextrin**  
Sodium hydroxide (105.7 g, 2.64 moles) was dissolved in 750 ml of distilled water and to this solution was added  $\beta$ -cyclodextrin (346.4 g of commercial preparation containing 13.4% of water; i.e., 300 g of anhydrous compound corresponding to 0.264 moles). Suspension was stirred at 60° C. until all  $\gamma$ -cyclodextrin was dissolved. Thereafter solution was cooled to room temperature and reflux condenser, filled with dry ice-acetone mixture, was attached. Then propylene oxide (redistilled; 185 ml; i.e., 153.5 g corresponding to 2.64 moles) was added, in fast manner but with necessary cautions, to the stirred solution. After addition of prop-

ylene oxide, the solution was stirred overnight at room temperature. Thereafter, the alkaline solution was neutralized by concentrated hydrochloric acid and evaporated in vacuo to the consistency of thick syrup. The syrup was dissolved in ethanol (1.5 L) and left standing to precipitate sodium chloride which was subsequently filtered off. The ethanolic solution was evaporated in vacuo and the rest was dissolved in water. The solution was enclosed in dialysis tubing and dialyzed for 2-3 hours against water. The dialyzed solution was then clarified by centrifugation and freeze-drying. The resulting white powder (282 g) contained 5.2% water (measured by weight loss), 0.65% of inorganic residue (by ashing), and 0.87% of chloride (elementary analysis). Upon prolonged standing water content increased to 7-8% but appearance of the compound (white powder) was not changed. By prolonged trituration of this powder with acetone, the products of self-condensation of propylene oxide, which contaminate the desired hydroxypropyl- $\beta$ -cyclodextrin, can be removed. The degree of substitution of the cyclodextrin moiety achieved in the above reaction was about 7; by variation of the amounts of reagents the substitution degrees were manipulated. Further or alternative purification of hydroxypropyl- $\beta$ -cyclodextrin consisted in the preparation of clear solutions of the raw material in water or ethanol and addition of large volumes of a nonpolar solvent (e.g., acetone) which precipitated the desired solid hydroxypropyl- $\beta$ -cyclodextrin, whereas highly substituted cyclodextrins and products of self-condensation of propylene oxide remained in the solution from which they can be recovered by evaporation, leaving oily liquid. In a representative experiment, precipitation yielded 81% of the desired solid hydroxypropyl- $\beta$ -cyclodextrin (degree of substitution 7 by nuclear magnetic resonance and 8.02 by mass spectroscopy) and 18% of oily liquid containing also hydroxypropyl- $\beta$ -cyclodextrin (degree of substitution 16 by nuclear magnetic resonance and 11.3 by mass spectrometry). When condensation of propylene oxide with  $\gamma$ -cyclodextrin was performed with a high ratio of the former to the latter, products were precipitable by cyclohexane but not by acetone and were semi-solid liquids (degree of substitution 14 by nuclear magnetic resonance).

## EXAMPLE 2

**Related preparations: O-alkylation of cyclodextrins with epoxides**

Basically the same conditions as above were used also for condensation of  $\alpha$ - or  $\gamma$ -cyclodextrins with propylene oxide; degrees of substitution achieved (by nuclear magnetic resonance) were close to those described above.

## EXAMPLE 3

**Related preparations: O-alkylation of cyclodextrins with organic halides.**

The procedures analogous to those above were also used for condensation of  $\beta$ -cyclodextrin with the following hydrophilic alkylating agents: (a) diethylaminoethylchloride-hydrochloride yielding diethylaminoethyl- $\beta$ -cyclodextrin; the degree of substitution in the product was about 3.5 (calculated from nitrogen content), (b) chloroacetic acid yielding carboxymethyl- $\beta$ -cyclodextrin; the degree of substitution in the product was about 4 (calculated from sodium content), (c) iodooctanamide yielding carboxamidomethyl- $\beta$ -

cyclodextrin; the degree of substitution in the product was about 3 (calculated from nitrogen content).

#### EXAMPLE 4

The solutions of solubilizer in water were saturated by stirring with excess of drug at room temperature for about 24 hours. The resulting suspensions were clarified either by filtration through a sintered glass filter or by centrifugation. Concentration of hormones in clarified solutions was measured spectrophotometrically. Representative results are given in Table 1, ante. In these experiments drugs were solubilized with cyclodextrin derivatives with constant degrees of substitution and which are given in the footnote to Table 1. In Table 2 are the results on the solubilization of sex hormones with hydroxypropyl- $\beta$ -cyclodextrin samples of different degrees of substitution. It is apparent that samples of hydroxypropyl- $\beta$ -cyclodextrin with medium degrees of substitution (5-7) are more effective solubilizers than those of higher degrees of substitution. It should be noted that while the former derivatives are solid and suitable for tableting purposes, the latter are semisolids or liquids. The results on the solubilization of sex hormones by cyclodextrin derivatives containing carboxamido or diethylaminoethyl or carboxymethyl substituents are summarized in Table 3. It is apparent that the carboxamido methyl derivative is a relatively potent solubilizer; nevertheless, comparison with the results in Table 2 show that the potency of hydroxypropyl containing derivatives has not been surpassed.

#### EXAMPLE 5

The above solutions of drugs in cyclodextrin derivatives were stable when kept at room temperature for several months and no microbial growth on the solutions was observed. When freeze-dried, the solutions which were prepared using solid derivatives of cyclodextrins again yielded non-hygroscopic, stable powders which were found suitable for tableting by direct compression. The tablets prepared in such a manner again dissolved completely in water as documented by Example 6.

#### EXAMPLE 6

Using a die of 0.9 cm diameter and a force of 3000 pounds weight exerted in a single-station hand-operated unit, the tablets were made directly from the freeze-dried solutions of drugs prepared as described above. In one such experiment tablets weighing about 130 mg and containing 10 mg of testosterone in complexed form were made. These tablets were stored for up to 16 months at room temperature in a well-closed glass container without any deterioration and were thereafter tested for dissolution properties. In the dissolution experiments a tablet was submerged in a basket made out of stainless steel mesh in a bath of water, at 20°C and sink conditions. As noted in FIG. 2, the complexed testosterone tablets dissolved completely within 4 minutes, whereas similar tablets prepared from testosterone and microcrystalline cellulose did not release practically any testosterone into the aqueous phase.

#### Definitions

The term "mixture of substituted amorphous cyclodextrins" is directed towards where there is more than one cyclodextrin and where there is more than one degree of substitution which will vary from a preferred range of about 3 to about 7.

#### I claim:

1. A method of producing a stabilizing amorphous complex of a drug and a mixture of cyclodextrins which comprises the steps of:

1. Dissolving an intrinsically amorphous mixture of cyclodextrin derivatives which are water soluble and capable of forming inclusion complexes with drugs in water; and
2. Solubilizing lipophilic drugs into the aqueous media to form a solution and form a solubilized drug/cyclodextrin complex.
2. A method of claim 1 wherein the solubilized complex is subjected to freeze-dried or evaporation to provide a solid cyclodextrin/drug complex in powder form.
3. A method of claim 1 wherein cyclodextrins used are substituted by at least one of the following substituents: hydroalkyl, carboxamide, diethylaminoethyl, carboxymethyl, and carboxyamidomethyl.
4. A method of claim 1 wherein the drug is a hormone.
5. A method of claim 4 wherein the drug is testosterone, an estrogen, or a progesterone.
6. A composition of matter which contains an amorphous complex of cyclodextrin and a drug.
7. A composition of matter of claim 6 wherein the drug is a hormone.
8. A composition of claim 6 wherein the drug is at least one of testosterone, progesterone, and an estrogenic drug.
9. A composition of matter of claim 6 in solid form.
10. A composition of matter of claim 9 which is a tablet.
11. A composition of matter of claim 6 which is in a liquid or semi-liquid form.
12. A composition of matter for use in the process of claim 1 containing a mixture of substituted cyclodextrin derivatives in amorphous form.
13. A composition of matter of claim 6 wherein the drug is a vitamin.
14. A composition of matter of claim 6 wherein the drug is a salt of retinoic acid.
15. A composition of matter of claim 6 wherein the drug is a steroid.
16. A composition of matter of claim 6 wherein the drug is a spiroinolactone.
17. A composition of matter of claim 6 wherein the drug is an antiviral agent.
18. A composition of matter of claim 17 wherein the antiviral agent is acyclovir.
19. A composition of matter of claim 6 wherein the drug is a diuretic.
20. A composition of matter of claim 19 wherein the diuretic is chlorothalidone.
21. A composition of matter of claim 6 wherein the drug is an anticoagulant.
22. A composition of matter of claim 21 wherein the anticoagulant is dicumolol.
23. A composition of matter of claim 6 wherein the drug is an anticonvulsant.
24. A composition of matter of claim 23 wherein the drug is diphenylhydantoin.
25. A composition of matter of claim 6 wherein the drug is an antiinflammatory agent.
26. A composition of matter of claim 25 wherein the antiinflammatory agent is iproniazid.
27. A composition of matter of claim 16 wherein the drug is oxyphenolol.
28. A composition of matter in solid or semi-solid form comprising at least one of testosterone, progesterone, and estradiol as an inclusion complex with poly- $\beta$ -cyclodextrin and/or hydroxypropyl- $\beta$ -cyclodextrin adapted for administration by buccal route.

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UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 4,727,064  
DATED : February 23, 1988  
INVENTOR(S) : Pitha, Josef

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Claim 1, line 1, change "stabilizing" to --stabilized--  
Claim 2, line 2, change "freeze-dried" to --freeze-drying--  
Claim 3, line 3, change "hydroalkyl" to --hydroxyalkyl--  
Claim 7, line 1, change "omposition" to --composition--

Signed and Sealed this  
Thirty-first Day of January, 1989

*Attest:*

DONALD J. QUIGG

*Attesting Officer*

*Commissioner of Patents and Trademarks*

# United States Patent [19]

Fontana

US00538432A

[11] Patent Number: 5,384,332

[45] Date of Patent: Jan. 24, 1995

[54] METHODS FOR INHIBITING AORTAL SMOOTH MUSCLE CELL PROLIFERATION AND RESTENOSIS WITH 1,1,2-TRIPHENYLBUT-1-ENE DERIVATIVES

[75] Inventor: Steven A. Fontana, Martinsville, Ind.

[73] Assignee: Eli Lilly and Company, Indianapolis, Ind.

[21] Appl. No.: 241,240

[22] Filed: May 11, 1994

[51] Int. Cl.<sup>6</sup> ..... A61K 31/135

[52] U.S. Cl. .... 514/648; 514/651;

514/824; 514/874

[58] Field of Search ..... 514/648

[56] References Cited

## U.S. PATENT DOCUMENTS

5,047,431 9/1991 Schickaneder et al. .... 514/648  
5,254,594 10/1993 Niikura et al. .... 514/648

Primary Examiner—Marianne M. Cintas

Assistant Examiner—T. J. Crieses

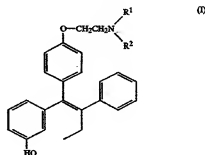
Attorney, Agent, or Firm—Steven A. Fontana; David E. Boone; Gerald V. Dahling

[57]

## ABSTRACT

The present invention provides novel methods of inhibiting aortal smooth muscle cell proliferation, particu-

larly restenosis, in humans, comprising administering to a human in need of treatment an effective amount of a compound of formula I



wherein

R<sup>1</sup> and R<sup>2</sup> may be the same or different provided that, when R<sup>1</sup> and R<sup>2</sup> are the same, each is a methyl or ethyl group, and, when R<sup>1</sup> and R<sup>2</sup> are different, one of them is a methyl or ethyl group and the other is a benzyl group, or a pharmaceutically acceptable salt thereof.

4 Claims, No Drawings

# METHODS FOR INHIBITING AORTAL SMOOTH MUSCLE CELL PROLIFERATION AND RESTENOSIS WITH 1,1,2-TRIPHENYLBUT-1-ENE DERIVATIVES

## BACKGROUND OF THE INVENTION

The present invention relates to the discovery that a group of 1,1,2-triphenylbut-1-ene derivatives are useful for inhibiting aortal smooth muscle cell proliferation, particularly restenosis, in humans.

Aortal smooth muscle cell proliferation plays an important role in diseases such as atherosclerosis and restenosis. Vascular restenosis after percutaneous transluminal coronary angioplasty (PTCA) has been shown to be a tissue response characterized by an early and late phase. The early phase, occurring hours to days after PTCA, is due to thrombosis with some vasospasms while the late phase appears to be dominated by excessive proliferation and migration of smooth muscle cells. In this disease, the increased cell motility and colonization by smooth muscle cells and macrophages contribute significantly to the pathogenesis of the disease. The excessive proliferation and migration of vascular smooth muscle cells may be the primary mechanism to the reocclusion of coronary arteries following PTCA, atherectomy, laser angioplasty, and arterial bypass graft surgery. See "Intimal Proliferation of Smooth Muscle Cells as an Explanation for Recurrent Coronary Artery Stenosis after Percutaneous Transluminal Coronary Angioplasty," Austin et al., *Journal of the American College of Cardiology* 8: 369-375 (Aug. 1985).

Vascular restenosis remains a major long term complication following surgical intervention of blocked arteries by percutaneous transluminal coronary angioplasty (PTCA), atherectomy, laser angioplasty, and arterial bypass graft surgery. In about 35% of the patients who undergo PTCA, reocclusion occurs within three to six months after the procedure. The current strategies for treating vascular restenosis include mechanical intervention by devices such as stents or pharmacologic therapies including heparin, low molecular weight heparin, coumadin, aspirin, fish oil, calcium antagonist, steroids, and prostacyclin. These strategies have failed to curb the reocclusion rate and have been ineffective for the treatment and prevention of vascular restenosis. See "Prevention of Restenosis after Percutaneous Transluminal Coronary Angioplasty: The Search for a 'Magic Bullet,'" Hermans et al., *American Heart Journal* 122: 171-187 (July 1991).

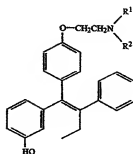
In the pathogenesis of restenosis, excessive cell proliferation and migration occurs as a result of growth factors produced by cellular constituents in the blood and the damaged arterial vessel wall which mediate the proliferation of smooth muscle cells in vascular restenosis.

Agents that inhibit the proliferation and/or migration of aortal smooth muscle cells are useful in the treatment and prevention of restenosis. The present invention provides for the use of compounds of formula I as aortal smooth muscle cell proliferation inhibitors.

## SUMMARY OF THE INVENTION

The present invention relates to methods for inhibiting aortal smooth muscle cell proliferation, particularly, restenosis, comprising administering to a human in need

of treatment an effective amount of a compound of formula I



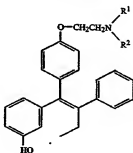
wherein

R<sup>1</sup> and R<sup>2</sup> may be the same or different provided that, when R<sup>1</sup> and R<sup>2</sup> are the same, each is a methyl or ethyl group, and, when R<sup>1</sup> and R<sup>2</sup> are different, one of them is a methyl or ethyl group and the other is a benzyl group, or a pharmaceutically acceptable salt thereof.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention concerns methods for inhibiting aortal smooth muscle cell proliferation, particularly restenosis, in humans. The "term inhibit" is defined to include its generally accepted meaning which includes prophylactically treating a subject from incurring one or more of these disease states, holding in check the symptoms of such a disease state, and/or treating such symptoms. Thus, the present methods include both medical therapeutic and/or prophylactic treatment, as appropriate.

The methods of this invention are practiced by administering to an individual in need of treatment an effective amount of a compound of formula I



wherein

R<sup>1</sup> and R<sup>2</sup> may be the same or different provided that, when R<sup>1</sup> and R<sup>2</sup> are the same, each is a methyl or ethyl group, and, when R<sup>1</sup> and R<sup>2</sup> are different, one of them is a methyl or ethyl group and the other is a benzyl group or a pharmaceutically acceptable salt thereof.

Compounds of formula I are known in the art and essentially are prepared via the methods described in U.S. Pat. No. 5,047,431, which is herein incorporated by reference.

A preferred formula I compound is that in which R<sup>1</sup> and R<sup>2</sup> each are methyl. This preferred compound is known as droloxifen which previously has been de-

scribed as an antiestrogenic agent and is useful for the treatment of hormone-dependent mammary tumors (U.S. Pat. No. 5,047,431), and for the relief of bone diseases caused by the deficiency of estrogen or the like (U.S. Pat. No. 5,254,594). Furthermore, droloxifen is known to have less of a uterotrophic effect than other antiestrogenic compounds such as tamoxifen.

Although the free-base form of formula I compounds can be used in the methods of the present invention, it is preferred to prepare and use a pharmaceutically acceptable salt form. Thus, the compounds used in the methods of this invention form pharmaceutically acceptable acid and base addition salts with a wide variety of inorganic and, preferably, organic acids and include the physiologically acceptable salts which are often used in pharmaceutical chemistry. Such salts are also part of this invention. Typical inorganic acids used to form such salts include hydrochloric, hydrobromic, hydroiodic, nitric, sulfuric, phosphoric, hypophosphoric, and the like. Salts derived from organic acids, such as aliphatic mono and dicarboxylic acids, phenyl substituted alkanic acids, hydroxyalkanoic and hydroxyalkandioic acids, aromatic acids, aliphatic and aromatic sulfonic acids, may also be used. Such pharmaceutically acceptable salts thus include acetate, phenylacetate, trifluoroacetate, ascorbate, benzoate, chlorobenzoate, dihalobenzoate, hydroxybenzoate, methoxybenzoate, methylbenzoate, *o*-acetoxybenzoate, naphthalene-2-benzoate, bromide, isobutyrate, phenylbutyrate,  $\beta$ -hydroxybutyrate, butyne-1,4-dioate, hexyne-1,4-dioate, caprate, caprylate, chloride, cinnamate, citrate, formate, fumarate, glycolate, heptanoate, hippurate, lactate, malate, maleate, hydroxymaleate, malonate, mandelate, mesylate, nicotinate, isonicotinate, nitrate, oxalate, phthalate, terephthalate, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate, propylate, propionate, phenylpropionate, salicylate, sebacate, succinate, suberate, sulfate, bisulfate, pyrosulfate, sulfite, bisulfite, sulfonate, benzenesulfonate, *p*-bromophenylsulfonate, chlorobenzenesulfonate, ethanesulfonate, 2-hydroxyethanesulfonate, methanesulfonate, naphthalene-1-sulfonate, naphthalene-2-sulfonate, *p*-toluenesulfonate, xylenesulfonate, tartrate, and the like. A preferred salt is the citrate salt.

The pharmaceutically acceptable acid addition salts are typically formed by reacting a compound of formula I with an equimolar or excess amount of acid. The reactants are generally combined in a mutual solvent such as diethyl ether or benzene. The salt normally precipitates out of solution within about one hour to 10 days and can be isolated by filtration or the solvent can be stripped off by conventional means.

The pharmaceutically acceptable salts of formula I compounds generally have enhanced solubility characteristics compared to the compound from which they are derived, and thus are often more amenable to formulation as liquids or emulsions.

Once prepared, the free base or salt form of formula I compounds can be administered to an individual in need of treatment for the methods herein described. The following nonlimiting test examples illustrate the methods of the present invention.

## Test Procedure

### General Preparation Procedure

In the examples illustrating the methods, a post-menopausal model is used to determine the effects of the different treatments upon test animal uteri.

Seventy-five day old female Sprague Dawley rats (weight range of 200 to 250 g) are obtained from Charles River Laboratories (Portage, Mich.). The animals are either bilaterally ovariectomized (OVX) or exposed to a Sham surgical procedure at Charles River Laboratories, and then shipped after one week. Upon arrival, they are housed in metal hanging cages in groups of 3 or 4 per cage and have ad libitum access to food (calcium content approximately 0.5%) and water for one week. Room temperature is maintained at 22.2 $\pm$ 1.7 $^{\circ}$  C, with a minimum relative humidity of 40%. The photoperiod in the room is 12 hours light and 12 hours dark.

### Dosing Regimen Tissue Collection.

After a one week acclimation period (therefore, two weeks post-OVX) daily dosing with test compound is initiated. 17 $\alpha$ -ethynyl estradiol and the test compounds are given orally, unless otherwise stated, as a suspension in 20% cyclodextrin. Animals are dosed daily for 4 days. Following the dosing regimen, animals are weighed and anesthetized with a ketamine xylazine (2:1, V/V) mixture and a blood sample is collected by cardiac puncture. The animals are then sacrificed by asphyxiation with CO<sub>2</sub>, the uterus is removed through a midline incision, and a wet uterine weight is determined. Inhibition of Vascular Smooth Cell Proliferation/Retenosis Test Procedure

Compounds of the present invention have capacity to inhibit aortal smooth cell proliferation. This can be demonstrated by using cultured smooth cells derived from rabbit aorta, proliferation being determined by the measurement of DNA synthesis. Cells are obtained by explant method as described in Ross, *J. of Cell Bio.* 50: 172 (1971). Cells are plated in 96 well microtiter plates for five days. The cultures become confluent and growth arrested. The cells are then transferred to Dulbecco's Modified Eagles Medium (DMEM) containing 0.5–2% platelet poor plasma, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mCi/ml  $^3$ H-thymidine, 20 ng/ml platelet-derived growth factor, and varying concentrations of the present compounds. Stock solution of the compounds is prepared in dimethyl sulphoxide and then diluted to appropriate concentration (0.01–30 mM) in the above assay medium. Cells are then incubated at 37 $^{\circ}$  C. for 24 hours under 5% CO<sub>2</sub>/95% air. At the end of 24 hours, the cells are fixed in methanol.  $^3$ H thymidine incorporation in DNA is then determined by scintillation counting as described in Bonin, et al., *Exp. Cell Res.* 181: 475–482 (1989).

Inhibition of aortal smooth muscle cell proliferation by the compounds of the present invention are further demonstrated by determining their effects on exponentially growing cells. Smooth muscle cells from rabbit aortae are seeded in 12 well tissue culture plates in DMEM containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. After 24 hours, the cells are attached and the medium is replaced with DMEM containing 10% serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and desired concentrations of the compounds. Cells are allowed to grow for four days.

Cells are treated with trypsin and the number of cells in each culture is determined by counting using a ZM-Coulter counter.

Activity in the above tests indicates that the compounds of the present invention are of potential in the treatment of aortal smooth muscle cell proliferation, particularly restenosis.

For the methods of the present invention, compounds of Formula I are administered continuously, from 1 to 4 times daily. In the case of restenosis, however, therapy may be limited to short (1-6 months) intervals following medical procedures such as angioplasty.

As used herein, the term "effective amount" means an amount of compound of the methods of the present invention which is capable of inhibiting the symptoms of the pathological conditions herein described. The specific dose of a compound administered according to this invention will, of course, be determined by the particular circumstances surrounding the case including, for example, the compound administered, the route of administration, the state of being of the patient, and the severity of the pathological condition being treated. A typical daily dose will contain a nontoxic dosage level of from about 0.25 mg to about 400 mg/day of a compound of the present invention. Preferred daily doses generally will be from about 1 mg to about 20 mg/day.

The compounds of this invention can be administered by a variety of routes including oral, rectal, transdermal, subcutaneous, intravenous, intramuscular, and intranasal. These compounds preferably are formulated prior to administration, the selection of which will be decided by the attending physician. Typically, a formula I compound, or a pharmaceutically acceptable salt thereof, is combined with a pharmaceutically acceptable carrier, diluent or excipient to form a pharmaceutical formulation.

The total active ingredients in such formulations comprises from 0.1% to 99.9% by weight of the formulation. By "pharmaceutically acceptable" it is meant the carrier, diluent, excipients, and/or salt must be compatible with the other ingredients of the formulation, and not deleterious to the recipient thereof.

Pharmaceutical formulations containing a compound of formula I can be prepared by procedures known in the art using well known and readily available ingredients. For example, the compounds of formula I can be formulated with common excipients, diluents, or carriers, and formed into tablets, capsules, suspensions, powders, and the like. Examples of excipients, diluents, and carriers that are suitable for such formulations include the following fillers and extenders such as starch, sugars, mannitol, and silicic derivatives binding agents such as carboxymethyl cellulose and other cellulose derivatives, alginates, gelatin, and polyvinyl-pyrrolidone; moisturizing agents such as glycerol; disintegrating agents such as calcium carbonate and sodium bicarbonate agents for retarding dissolution such as paraffin resorption accelerators such as quaternary ammonium compounds; surface active agents such as cetyl alcohol, glycerol monostearate; adsorptive carriers such as kaolin and bentonite; and lubricants such as talc, calcium and magnesium stearate, and solid polyethyl glycols.

The compounds also can be formulated as elixirs or solutions for convenient oral administration or as solutions appropriate for parenteral administration, for example, by intramuscular, subcutaneous or intravenous routes.

Additionally, the compounds are well suited to formulation as sustained release dosage forms and the like. The formulations can be so constituted that they release the active ingredient only or preferably in a particular physiological location, possibly over a period of time. The coatings, envelopes, and protective matrices may be made, for example, from polymeric substances or waxes.

Compounds of formula I generally will be administered in a convenient formulation. The following formulation examples only are illustrative and are not intended to limit the scope of the present invention. cI Formulations

In the formulations which follow, "active ingredient" means a compound of formula I, or a salt thereof.

Formulation 1: Gelatin Capsules

Hard gelatin capsules are prepared using the following:

Ingredient	Quantity (mg/capsule)
Active ingredient	0.25-400
Starch, NF	0-650
Starch flowable powder	0-50
Silicone fluid 150 centistokes	0-15

The formulation above may be changed in compliance with the reasonable variations provided.

A tablet formulation is prepared using the ingredients below:

Formulation 2: Tablets

Ingredient	Quantity (mg/tablet)
Active ingredient	0.25-400
Cellulose, microcrystalline	200-650
Silicon dioxide, fumed	10-550
Stearate acid	5-15

The components are blended and compressed to form tablets.

Alternatively, tablets each containing 0.25-400 mg of active ingredient are made up as follows:

Formulation 3: Tablets

Ingredient	Quantity (mg/tablet)
Active ingredient	0.25-400
Starch	45
Cellulose, microcrystalline	35
Polyvinylpyrrolidone	4
(as 10% solution in water)	
Sodium carboxymethyl cellulose	4.5
Magnesium stearate	0.5
Talc	1

The active ingredient, starch, and cellulose are passed through a No. 45 mesh U.S. sieve and mixed thoroughly. The solution of polyvinylpyrrolidone is mixed with the resultant powders which are then passed through a No. 14 mesh U.S. sieve. The granules so produced are dried at 50°-60° C. and passed through a No. 18 mesh U.S. sieve. The sodium carboxymethyl starch, magnesium stearate, and talc, previously passed through a No. 60 U.S. sieve, are then added to the granules which, after mixing, are compressed on a tablet machine to yield tablets.

Suspensions each containing 0.25-400 mg of medication per 5 ml dose are made as follows:

Formulation 4: Suspensions

Ingredient	Quantity (mg/5 mL)
Active ingredient	0.25-400 mg
Sodium carboxymethyl cellulose	20 mg
Syrup	1.25 mg
Benzoic acid solution	0.10 mL
Flavor	q.v.
Color	q.v.
Purified water to	5 mL

The medicament is passed through a No. 45 mesh U.S. sieve and mixed with the sodium carboxymethyl cellulose and syrup to form smooth paste. The benzoic acid solution, flavor, and color are diluted with some of the water and added, with stirring. Sufficient water is then added to produce the required volume.

An aerosol solution is prepared containing the following ingredients:

Formulation 5: Aerosol

Ingredient	Quantity (% by weight)
Active ingredient	0.25
Ethanol	25.75
Propellant 22 (Chlorodifluoromethane)	70.00

The active ingredient is mixed with ethanol and the mixture added to a portion of the propellant 22, cooled to 30° C., and transferred to a filling device. The required amount is then fed to a stainless steel container and diluted with the remaining propellant. The valve units are then fitted to the container.

Suppositories are prepared as follows:

Formulation 6: Suppositories

Ingredient	Quantity (mg/suppository)
Active ingredient	250
Saturated fatty acid glycerides	2,000

The active ingredient is passed through a No. 60 mesh U.S. sieve and suspended in the saturated fatty acid glycerides previously melted using the minimal necessary heat. The mixture is then poured into a suppository mold of nominal 2 g capacity and allowed to cool.

An intravenous formulation is prepared as follows:

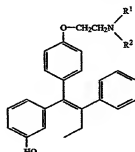
Formulation 7: Intravenous Solution

Ingredient	Quantity
Active ingredient	20 mg
Isotonic saline	1,000 mL

The solution of the above ingredients is intravenously administered to a patient at a rate of about 1 mL per minute.

I claim:

1. A method for inhibiting aortal smooth muscle cell proliferation comprising administering to a human in need of treatment an effective amount of a compound of formula I

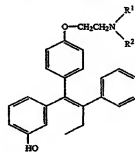


wherein

R<sup>1</sup> and R<sup>2</sup> may be the same or different provided that, when R<sup>1</sup> and R<sup>2</sup> are the same, each is a methyl or ethyl group, and, when R<sup>1</sup> and R<sup>2</sup> are different, one of them is a methyl or ethyl group and the other is a benzyl group;

or a pharmaceutically acceptable salt thereof.

2. A method for inhibiting restenosis comprising administering to a human in need of treatment an effective amount of a compound formula I



wherein

R<sup>1</sup> and R<sup>2</sup> may be the same or different provided that, when R<sup>1</sup> and R<sup>2</sup> are the same, each is a methyl or ethyl group, and, when R<sup>1</sup> and R<sup>2</sup> are different, one of them is a methyl or ethyl group and the other is a benzyl group;

or a pharmaceutically acceptable salt thereof.

3. A method according to claim 2 wherein the compound of formula I is a compound wherein R<sup>1</sup> and R<sup>2</sup> each are methyl, or a pharmaceutically acceptable salt thereof.

4. A method according to claim 3 wherein said salt thereof is the citrate salt.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
CERTIFICATE OF CORRECTION

PATENT NO. :5,384,332  
DATED :January 24, 1995  
INVENTOR(S) :Steven A. Fontana

It is certified that an error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 1, line 44, "weight heparin, coumarin," and should read, -- weight heparin, coumarin,--

Column 5, line 52, "the following fillers and extenders such as starch," and should read, -- the following: fillers and extenders such as starch, --

Column 5 line 53 "mannitol, and silicic derivatives binding agents such" and should read -- mannitol, and silicic derivatives; binding agents such --

Column 5, lines 57-58, "sodium bicarbonate agents, " and should read, -- sodium bicarbonate; agents --

column 5, line 58, "dissolution such as paraffin" and should read, -- dissolution such as paraffin; --

Column 6, line 12, "of the present invention. cl," should read, -- of the present invention. --

Signed and Sealed this  
Fifth Day of September, 1995

Attest:



Attesting Officer

BRUCE LEHMAN  
Commissioner of Patents and Trademarks



US006117911A

## United States Patent [19]

Grainger et al.

[11] Patent Number: 6,117,911

[45] Date of Patent: Sep. 12, 2000

## [54] COMPOUNDS AND THERAPIES FOR THE PREVENTION OF VASCULAR AND NON-VASCULAR PATHOLOGIES

[75] Inventors: David J. Grainger; James C. Metcalfe, both of Cambridge, United Kingdom; Sudhakar Kasina, Mercer Island, Wash.

[73] Assignee: NeoRx Corporation, Seattle, Wash.

[21] Appl. No.: 09/057,323

[22] Filed: Apr. 9, 1998

Related U.S. Application Data

[60] Provisional application No. 60/043,852, Apr. 11, 1997.

[51] Int. Cl.<sup>7</sup> A61K 31/135; C07C 213/00

[52] U.S. Cl. 514/648; 564/317

[58] Field of Search 514/648; 564/317

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Primary Examiner—Deborah C. Lambkin  
Attorney, Agent, or Firm—Schwegman, Lundberg, Woessner & Kluth, P.A.

## [57] ABSTRACT

The invention provides a method of treating a mammal having, or at risk of, an indication associated with a TGF-beta deficiency comprising administering one or more agents that is effective to elevate the level of TGF-beta. The invention also provides novel compounds that elevate TGF-beta levels, as well as pharmaceutical compositions comprising compounds that elevate TGF-beta levels, and methods for detecting diseases associated with endothelial cell activation.

18 Claims, 14 Drawing Sheets

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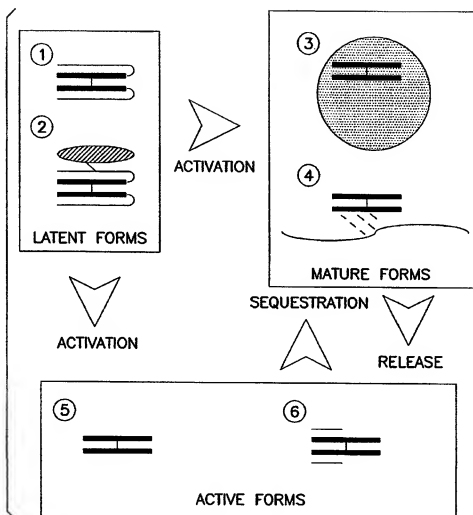


FIG. 1

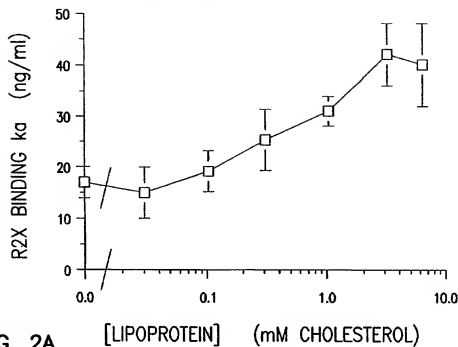


FIG. 2A

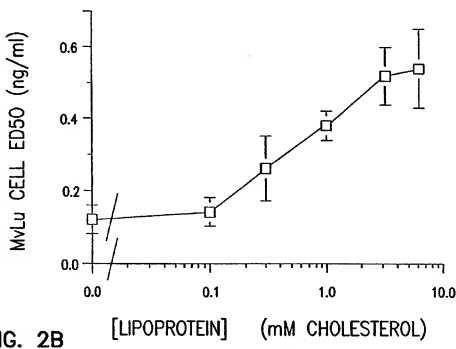


FIG. 2B

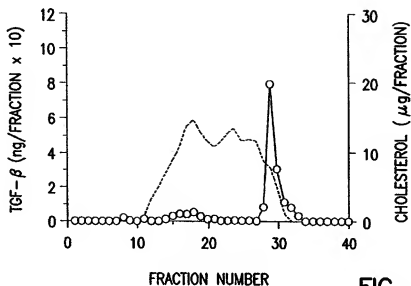


FIG. 3A

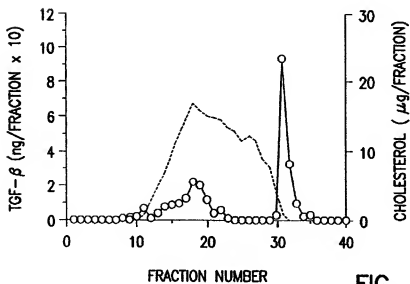


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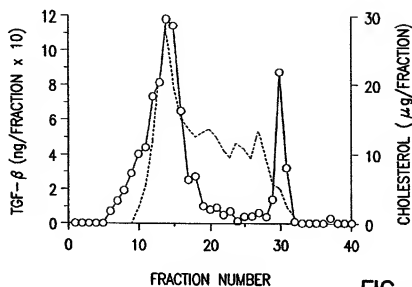


FIG. 3C

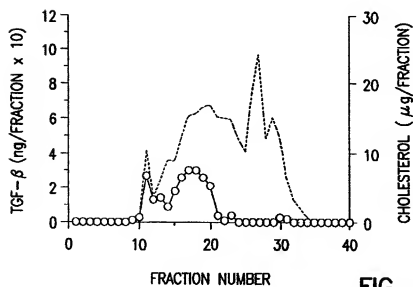


FIG. 3D

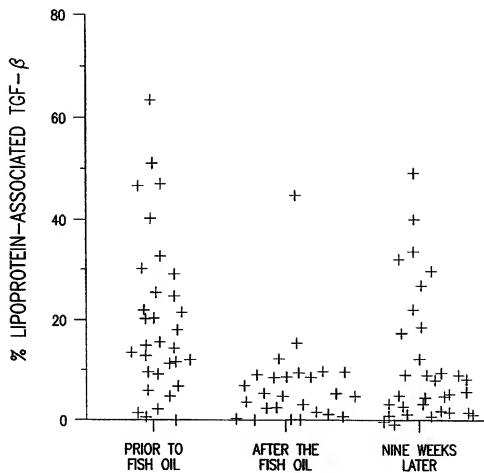


FIG. 4

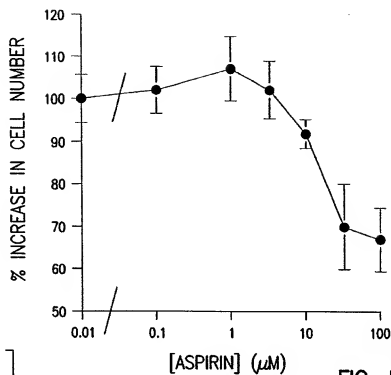


FIG. 5A

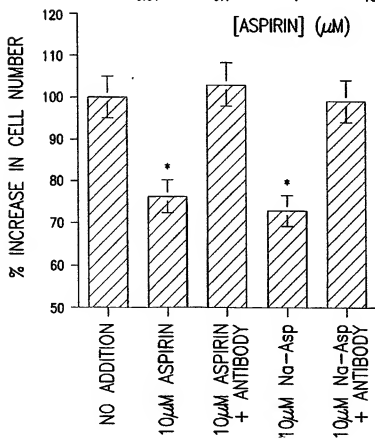


FIG. 5B

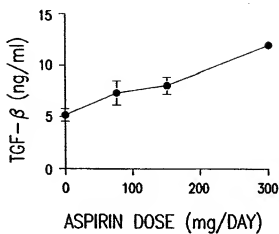


FIG. 6A

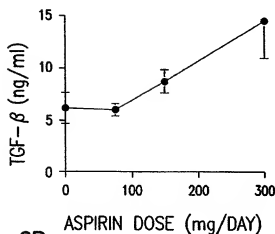


FIG. 6B

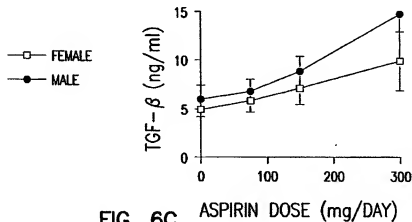


FIG. 6C

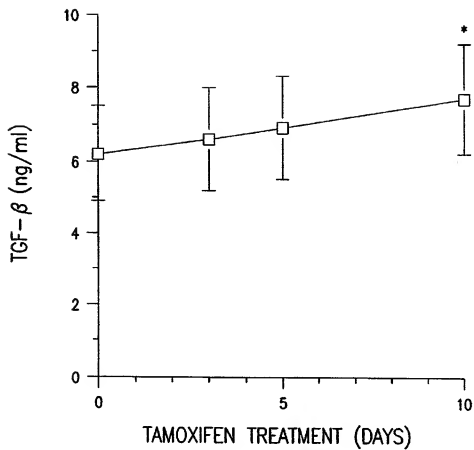


FIG. 7

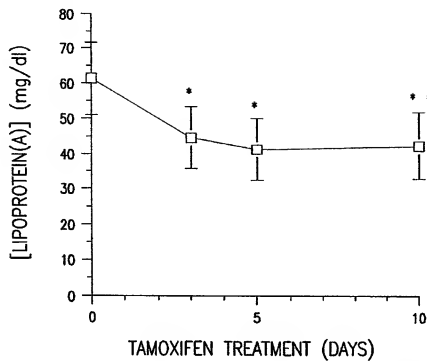


FIG. 8A

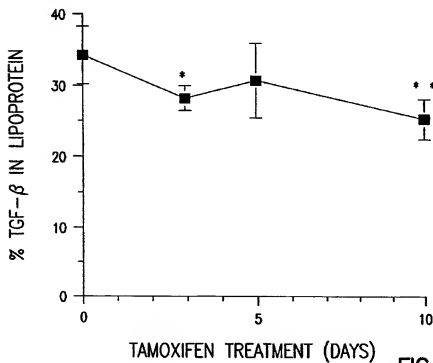


FIG. 8B

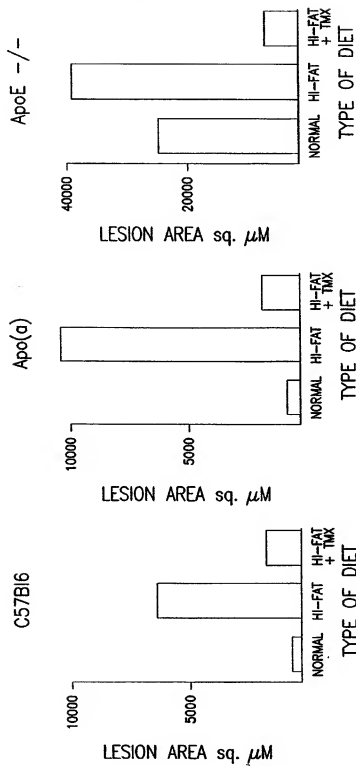


FIG. 9

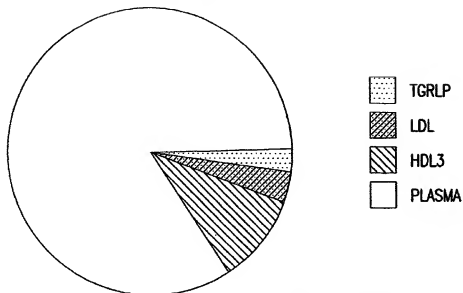


FIG. 10A

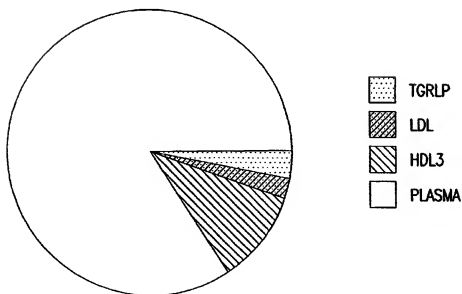


FIG. 10B

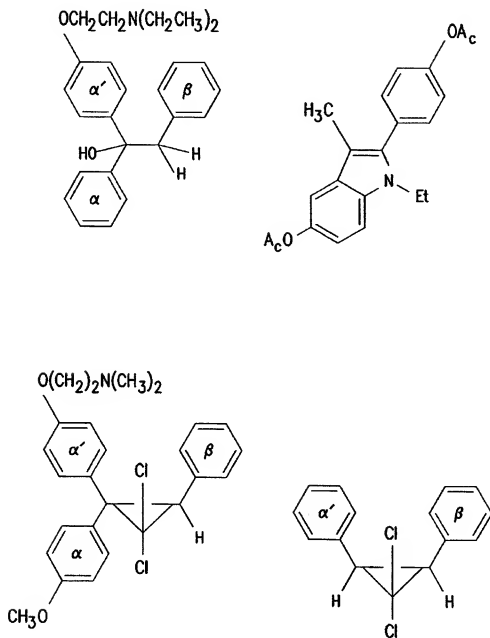


FIG. 11

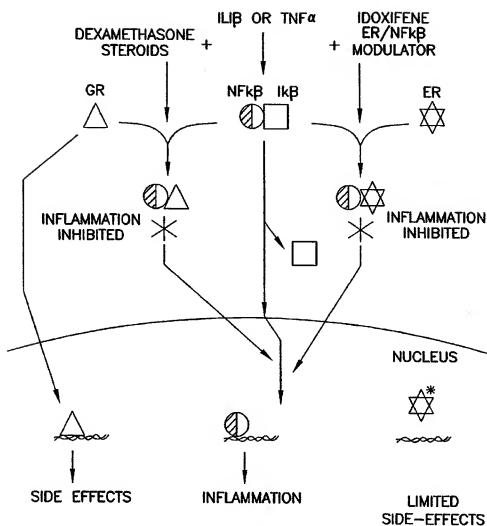
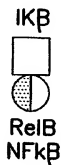


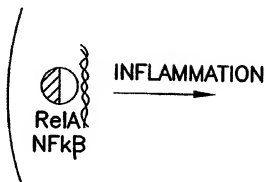
FIG. 12

INFLAMMATION + NO DRUG



IKB  
RelB  
NFkB

The diagram shows a square labeled IKB bound to a circle. The circle is divided vertically: the left half is shaded with a stippled pattern and labeled RelB, and the right half is unshaded and labeled NFkB.

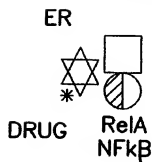


RelA  
NFkB

INFLAMMATION

The diagram shows a circle divided vertically: the left half is shaded with diagonal lines and labeled RelA, and the right half is unshaded and labeled NFkB. This circle is bound to a DNA double helix. An arrow points from this complex to the word INFLAMMATION.

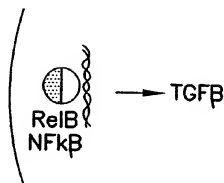
INFLAMMATION + DRUG



ER  
DRUG

RelA  
NFkB

The diagram shows a square labeled ER bound to a circle. The circle is divided vertically: the left half is shaded with diagonal lines and labeled RelA, and the right half is unshaded and labeled NFkB. To the left of the square is a six-pointed star labeled DRUG with an asterisk below it.



RelB  
NFkB

TGFb

The diagram shows a circle divided vertically: the left half is shaded with a stippled pattern and labeled RelB, and the right half is unshaded and labeled NFkB. This circle is bound to a DNA double helix. An arrow points from this complex to the label TGFb.

INHIBITS  
INFLAMMATION

FIG. 13

# 1

## COMPOUNDS AND THERAPIES FOR THE PREVENTION OF VASCULAR AND NON-VASCULAR PATHOLOGIES

### PRIORITY OF INVENTION

This application claims priority under 35 U.S.C. §119(e) from U.S. Provisional Patent Application Ser. No. 60/043,852, filed Apr. 11, 1997.

### BACKGROUND OF THE INVENTION

TGF-beta dynamically regulates the differentiation of smooth muscle cells, and has been postulated to maintain vessel wall structure. TGF-beta also appears to possess immunosuppressive properties which protect the vascular endothelium against local inflammation and damage. Moreover, TGF-beta may inhibit the proliferation and migration of smooth muscle cells after vascular injury.

TGF-beta is synthesized as a latent peptide (FIG. 1). Latent TGF-beta refers to any of several complexes that include the 25 kD TGF-beta dimer in association with the latency associated peptide (LAP) or any of several additional TGF-beta binding proteins (LTBPs). Latent TGF-beta has no biological activity, i.e., it does not bind to the TGF-beta receptors.

The 25 kD TGF-beta dimer is also found associated with matrix components or other plasma proteins (FIG. 1). TGF-beta that is associated with matrix components or other plasma proteins is termed mature TGF-beta. This association also prevents the binding of TGF-beta to the TGF-beta receptors.

In addition to latent and mature forms of TGF-beta, which cannot bind to the TGF-beta receptors and which possess no known biological activity, TGF-beta also exists in forms which are capable of binding to the TGF-beta receptors and which elicit biological effects (FIG. 1). These forms of TGF-beta are termed "active TGF-beta." One example of a form of active TGF-beta is the 25 kD TGF-beta dimer which is free from association with LAP/LTBPs, or matrix or plasma components. The process(es) by which the latent form of TGF-beta is converted to the active form is termed "activation." The process(es) by which the mature form of TGF-beta is converted to the active form is termed "release."

Decreased levels of TGF-beta have been implicated in the development of atherosclerosis. Atherosclerosis is a disease of the major arteries, typified by changes in the vessel wall architecture. At lesion-prone sites where the endothelium becomes damaged or dysfunctional, smooth muscle cells from the media of the vessel migrate into the intima. At these sites, leukocytes, and in particular, monocytes and macrophages invade the expanded intima. As the lesion develops, lipid from the circulation is deposited into the intima (reviewed in Ross, *Nature*, 362, 801 (1993); Grainger et al. *Biol. Rev. Camb. Philos. Soc.*, 70, 571 (1995)).

Agents which elevate TGF-beta activity, such as tamoxifen (TMX) (Grainger et al., *Biochem. J.*, 294 109 (1993)) and aspirin (Grainger et al., *Nat. Med.*, 1, 74 (1995)), can exhibit cardioprotective effects. However, the positive cardioprotective effects of these agents may be counterindicated by their potential side effects. TMX can cause liver carcinogenicity in rats, has been correlated with an increased risk of endometrial cancer in women and may increase the risk of certain gut cancers. Aspirin may result in ulcerogenesis and increased bleeding.

Agents which elevate TGF-beta levels may also be useful to prevent or treat diseases or conditions including cancer,

Marfan's syndrome, Parkinson's disease, fibrosis, Alzheimer's disease, senile dementia, osteoporosis, diseases associated with inflammation, such as rheumatoid arthritis, multiple sclerosis and lupus erythematosus, and other autoimmune disorders. Such agents may also be useful to promote wound healing and to lower serum cholesterol levels.

Thus, there is a need for improved therapeutic methods and agents useful to maintain or elevate TGF-beta levels in mammals.

### SUMMARY OF THE INVENTION

The present invention provides a method to maintain or elevate TGF-beta levels in a mammal, such as a human, in need of such therapy. The method comprises administering an effective amount of an aspirinate as defined herein. The method can also be carried out by administering an amount of a first therapeutic agent effective to elevate the level of latent TGF-beta and an amount of a second therapeutic agent effective to increase the level of TGF-beta which is capable of binding to the TGF-beta receptors, wherein said amounts are effective to maintain or elevate the level of TGF-beta in said mammal.

The invention also provides a method of preventing or treating a mammal, such as a human, having, or at risk of, a vascular indication which is associated with a TGF-beta deficiency. The method comprises the administration of an amount of an aspirinate that elevates the level of TGF-beta in said mammal so as to inhibit or reduce diminution in vessel lumen diameter. Preferably, the levels of active TGF-beta are elevated after administration of the aspirinate.

Preferred agents useful in the practice of the invention are copper aspirinates. Preferably, the effective amount of aspirinate inhibits lipid accumulation, increases plaque stability, decreases lesion formation or development, promotes lesion regression, or any combination thereof. Agents useful in the practice of the method include aspirinate salts such as copper salts of aspirinates, including copper aspirinate itself (copper 2-acetylsalicylate or copper 2-acetoxybenzoate), salicylate salts such as copper salts of salicylates, including copper salicylate (copper 2-hydroxybenzoate), or a compound of formula (I) (see below) including a pharmaceutically acceptable salt thereof, or a combination thereof.

An aspirinate useful in the present invention is a compound of formula (I):



(I)

wherein

R<sup>1</sup> is hydrogen, halo, nitro, cyano, hydroxy, CF<sub>3</sub>, —NR<sub>2</sub>R<sub>3</sub>, —C(=O)OR<sub>2</sub>, —C(=N)OR<sub>2</sub>, —OC(=O)OR<sub>2</sub>, (C<sub>1</sub>–C<sub>6</sub>)alkyl or (C<sub>1</sub>–C<sub>6</sub>)alkoxy;

R<sup>2</sup> is hydrogen or —XR<sub>3</sub>;

R<sup>3</sup> is —C(=O)YR<sub>4</sub> or —N(R<sub>2</sub>)C(=O)R<sub>4</sub>—;

R<sup>4</sup> is —(O)R<sub>5</sub> or R<sup>4</sup> is (C<sub>1</sub>–C<sub>6</sub>)alkyl, (C<sub>1</sub>–C<sub>6</sub>)alkenyl or (C<sub>1</sub>–C<sub>6</sub>)alkynyl;

R<sup>5</sup> is hydrogen, —C(=O)OR<sub>6</sub> or —C(=O)SR<sub>6</sub>;

n is 0, 1 or 2;

X is oxygen,  $-\text{N}(\text{R}_1)-$ , or sulfur;

Y is oxygen or sulfur;

$\text{R}_3$  is  $(\text{C}_1-\text{C}_6)$ alkanoyl,  $(\text{C}_1-\text{C}_6)$ alkyl, or hydrogen;

$\text{R}_6$  is hydrogen or  $(\text{C}_1-\text{C}_6)$ alkyl;

$\text{R}_6$  and  $\text{R}_7$  are each independently hydrogen,  $(\text{C}_1-\text{C}_6)$ alkyl, phenyl,  $\text{C}(=\text{O})\text{OH}$ ,  $\text{C}(=\text{O})\text{O}(\text{C}_1-\text{C}_6)$ alkyl,  $\text{CH}_2\text{C}(=\text{O})\text{OH}$ ,  $\text{CH}_2\text{C}(=\text{O})\text{O}(\text{C}_1-\text{C}_6)$ alkyl, or  $(\text{C}_1-\text{C}_6)$ alkoxy; or  $\text{R}_6$  and  $\text{R}_7$  together with the nitrogen to which they are attached are a 3, 4, 5, or 6 membered heterocyclic ring; and

$\text{R}_1-\text{R}_4$  are independently hydrogen or  $(\text{C}_1-\text{C}_6)$ alkyl;

a pharmaceutically acceptable salt thereof; or a combination thereof,

provided that  $\text{R}^2$  and  $\text{R}^3$  are on adjacent positions of the ring to which they are attached, or are on the 2- and 5-positions of the ring; and further provided that when  $\text{R}^2$  is hydrogen;  $\text{R}^3$  is on the 2- or 5-position of the ring to which it is attached and  $\text{R}^4$  is  $(\text{C}_1-\text{C}_6)$ alkoxy. Preferably, the compound of formula (I) is not 3-acetoxy-2-carboxythiophene.

Also provided is a method of preventing or treating a mammal having, or at risk of, a vascular indication by administering there to an amount of a first therapeutic agent and an amount of a second therapeutic agent which together are effective to elevate the level of TGF-beta, preferably the level of active TGF-beta, in said mammal. Preferably, the administration inhibits or reduces diminution in vessel lumen diameter. The inhibition or reduction in diminution in vessel lumen diameter preferentially occurs at a site in a vessel where the vascular indication is, or is likely to be, manifested. The invention thus provides for combination therapy, e.g., the administration of one agent that can elevate the level of latent TGF-beta, and another agent that can elevate the level of TGF-beta which is available to bind to, or is capable of binding to, the TGF-beta receptor. This combination therapy can yield a significantly greater cardiovascular efficacy than would be expected from the administration of either agent singly. The therapeutic agents can act in a synergistic, rather than in an additive, manner to elevate TGF-beta levels. The therapeutic agents can be administered simultaneously in a single dosage form simultaneously in individual dosages, or sequentially.

A first therapeutic agent useful in this embodiment of the invention includes an aspirinate, e.g., a compound of formula (I). Another preferred first therapeutic agent comprises a compound of formula VI (see below). A preferred second therapeutic agent useful in this embodiment of the invention comprises at least one omega-3 fatty acid, which can be provided, e.g., by dosages of fish oil. Another preferred second therapeutic agent is selected from at least one compound of formula VI. Thus, a compound of formula VI may both elevate latent levels of TGF-beta and elevate the levels of TGF-beta which can bind to the TGF-beta receptors. Preferably, the combination of the therapeutic agents inhibits lipid accumulation, increases plaque stability, decreases lesion formation or development, promotes lesion regression, or any combination thereof.

A compound useful in the present invention is a compound of formula (VI):

(VI)



wherein

$\text{R}^4$  is  $(\text{C}_1-\text{C}_6)$ alkyl, or aryl, optionally substituted by 1, 2, or 3  $\text{V}$ ;

$\text{R}^7$  is phenyl, optionally substituted by 1, 2, or 3  $\text{V}$ ; or  $\text{R}^7$  is  $(\text{C}_1-\text{C}_{12})$ alkyl, halo $(\text{C}_1-\text{C}_{12})$ alkyl,  $(\text{C}_1-\text{C}_6)$ cycloalkyl,  $(\text{C}_1-\text{C}_6)$ alkylcyclo $(\text{C}_1-\text{C}_6)$ alkyl,  $(\text{C}_1-\text{C}_6)$ cycloalkenyl, or  $(\text{C}_1-\text{C}_6)$ alkyl $(\text{C}_1-\text{C}_6)$ cycloalkenyl;

$\text{R}^6$  is hydrogen or phenyl, optionally substituted at the 2-position with  $\text{R}_7$ , and optionally substituted by 1, 2, or 3  $\text{V}$ ;

$\text{R}^5$  is hydrogen, nitro, halo, aryl, heteroaryl, aryl $(\text{C}_1-\text{C}_6)$ alkyl, heteroaryl $(\text{C}_1-\text{C}_6)$ alkyl, halo $(\text{C}_1-\text{C}_{12})$ alkyl, cyano $(\text{C}_1-\text{C}_{12})$ alkyl,  $(\text{C}_1-\text{C}_6)$ alkoxy, carbonyl $(\text{C}_1-\text{C}_6)$ alkyl,  $(\text{C}_1-\text{C}_{12})$ alkyl,  $(\text{C}_1-\text{C}_6)$ cycloalkyl,  $(\text{C}_1-\text{C}_6)$ alkylcyclo $(\text{C}_1-\text{C}_6)$ alkyl,  $(\text{C}_1-\text{C}_6)$ cycloalkenyl, or  $(\text{C}_1-\text{C}_6)$ alkyl $(\text{C}_1-\text{C}_6)$ cycloalkenyl, wherein any aryl or heteroaryl may optionally be substituted by 1, 2, or 3,  $\text{V}$ ; or

$\text{R}^5$  and  $\text{R}_7$  together are  $-\text{CH}_2\text{CH}_2-$ ,  $-\text{S}-$ ,  $-\text{O}-$ ,  $-\text{N}(\text{H})-$ ,  $-\text{N}(\text{C}_1-\text{C}_6)$ alkyl-,  $-\text{OCH}_2-$ ,  $-\text{OC}[(\text{C}_1-\text{C}_6)$ alkyl]-, or  $-\text{CH}=\text{CH}-$ ;

$-\text{}$  is a single bond or is  $-\text{C}(\text{B})(\text{D})-$ , wherein B and D are each independently hydrogen,  $(\text{C}_1-\text{C}_6)$ alkyl, or halo;

$\text{V}$  is  $\text{OPO}_3\text{H}_2$ ,  $(\text{C}_1-\text{C}_6)$ alkyl,  $(\text{C}_1-\text{C}_6)$ alkoxy, mercapto,  $(\text{C}_1-\text{C}_6)$ alkylthio, halo, trifluoromethyl, pentafluoroethyl, nitro,  $\text{N}(\text{R}_1)(\text{R}_2)$ , cyano, trifluoromethoxy, pentafluoromethoxy, benzoyl, hydroxy,  $-(\text{CH}_2)_0-4\text{C}(=\text{O})(\text{C}_1-\text{C}_6)$ alkyl,  $-\text{UC}(=\text{O})(\text{C}_1-\text{C}_6)$ alkyl, benzyl,  $-\text{OSO}_2(\text{CH}_2)_0-4\text{CH}_3$ ,  $-\text{U}(\text{CH}_2)_0-4\text{COOR}$ ,  $-(\text{CH}_2)_0-4\text{COOR}$ ,  $-\text{U}(\text{CH}_2)_0-4\text{OR}$ ,  $-(\text{CH}_2)_0-4\text{OR}$ ,  $-\text{U}(\text{CH}_2)_0-4\text{C}(=\text{O})\text{R}$ ,  $-(\text{CH}_2)_0-4\text{C}(=\text{O})\text{R}$ ,  $-\text{U}(\text{CH}_2)_0-4\text{R}$ ,  $-(\text{CH}_2)_0-4\text{R}$ , or  $-\text{U}(\text{CH}_2)_0-4\text{OC}(=\text{O})\text{R}$ , wherein U is O,  $\text{N}(\text{R}_1)$ , or S; Z is  $-(\text{CH}_2)_0-4$ ,  $-\text{O}-$ ,  $-\text{OCH}_2-$ ,  $-\text{CH}_2\text{O}-$ ,  $-\text{C}(=\text{O})\text{O}-$ ,  $-\text{N}(\text{R}_1)-$ ,  $-\text{CmO}-$ , or a covalent bond;

$\text{R}_1$  is amino, optionally substituted with one or two  $(\text{C}_1-\text{C}_6)$ alkyl; or an N-heterocyclic ring optionally containing 1 or 2 additional  $\text{N}(\text{R}_1)$ , S, or nontoxic  $\text{O}$ , wherein  $\text{R}^1$  is H  $(\text{C}_1-\text{C}_6)$ alkyl, phenyl, or benzyl;

$\text{R}_6$  and  $\text{R}_7$  are independently hydrogen,  $(\text{C}_1-\text{C}_6)$ alkyl, phenyl, benzyl, or  $(\text{C}_1-\text{C}_6)$ alkanoyl; or  $\text{R}_6$  and  $\text{R}_7$  together with the nitrogen to which they are attached are a 3, 4, 5, or 6 membered heterocyclic ring;

$\text{R}_6$  is H or  $(\text{C}_1-\text{C}_6)$ alkyl; and

$\text{R}_6$  and  $\text{R}_7$  are independently hydrogen,  $(\text{C}_1-\text{C}_6)$ alkyl, phenyl, benzyl, or  $(\text{C}_1-\text{C}_6)$ alkanoyl;

the compound is MER25;

or a pharmaceutically acceptable salt thereof.

As described hereinbelow, the combination of aspirin plus an agent such as fish oil that increases the level of TGF-beta which is capable of binding to the TGF-beta receptors, results in a greater reduction in lesion formation in apoE knockout mice relative to aspirin or fish oil therapy alone. Surprisingly, the combination of aspirin and fish oil, which

comprises a plurality of omega-3 fatty acids, exerts a markedly synergistic, rather than an additive, effect. Thus, a combination of an agent that elevates the level of latent TGF-beta, e.g., low doses of aspirin or an aspirinate, with an agent that increases the level of TGF-beta which can bind to its receptor, e.g., at least one omega-3 fatty acid, can be very effective in preventing or treating vascular disease. As used herein, "at least one" omega-3 fatty acid reflects the fact that one of skill in the art would recognize that natural sources of omega-3 fatty acids contain a plurality, about 1 to 30, preferably about 1 to 25, and more preferably about 2 to 20, of omega-3 fatty acids.

Another embodiment of the invention is a method for preventing atherosclerosis in a mammal at risk therefor, or treating atherosclerosis in a mammal, by administering to the mammal an amount of a first therapeutic agent and an amount of a second therapeutic agent effective to maintain or elevate the level of TGF-beta. The first therapeutic agent preferably increases the level of latent TGF-beta, e.g., is aspirin or an aspirinate, or a combination thereof, and the second therapeutic agent increases the level of TGF-beta which is capable of binding to the TGF-beta receptors. Thus, the agents of the invention are administered in a combined amount that prevents or inhibits diminution in vessel lumen diameter at, or near, a site or potential site of atherosclerotic lesion formation or development. A preferred first therapeutic agent comprises aspirin or an aspirinate. A preferred second therapeutic agent comprises at least one omega-3 fatty acid.

The invention also provides a method to inhibit diminution in mammalian vessel lumen diameter. The method comprises administering to a mammal in need of said therapy, an amount of a first therapeutic agent and an amount of a second therapeutic agent effective to maintain or elevate the level of TGF-beta, so as to inhibit or reduce vessel lumen diminution. The inhibition or reduction in diminution in vessel lumen diameter preferentially occurs at a site in a vessel where the diminution is or is likely to be manifested. The first therapeutic agent increases the level of latent TGF-beta, with the proviso that the first therapeutic agent is not aspirin. The first therapeutic agent is preferably an aspirinate. The second therapeutic agent increases the level of TGF-beta which is capable of binding to the TGF-beta receptors.

Also provided is a combination therapy to maintain or elevate TGF-beta levels in a mammal in need of such treatment. The method comprises the administration of an amount of a first therapeutic agent and a second therapeutic agent, wherein said amount is effective to maintain or elevate the level of TGF-beta. The first therapeutic agent increases the level of latent TGF-beta, while the second therapeutic agent increases the level of TGF-beta which is capable of binding to the TGF-beta receptors. A preferred first therapeutic agent comprises aspirin or an aspirinate, while a preferred second therapeutic agent comprises at least one omega-3 fatty acid.

The invention also provides a method to maintain or elevate TGF-beta levels in a mammal in need of such treatment. The method comprises the administration of an amount of an aspirinate effective to maintain or elevate the level of TGF-beta, preferably active TGF-beta, in said mammal.

The invention also provides a method of preventing or treating a mammal having, or preventing in a mammal at risk of, a condition which is associated with a TGF-beta deficiency. Also provided is a method to maintain TGF-beta levels in a mammal. The methods comprise the administra-

tion of one or more agents in an amount effective to elevate or maintain the level of TGF-beta in said mammal. The effective amount of the agent or agents may increase the level of latent TGF-beta or the level of TGF-beta which is capable of binding to the TGF-beta receptors. Agents useful to increase the level of latent TGF-beta include, but are not limited to, idoxifene, toremifene, raloxifene, droloxifene, ethinyl estradiol, diethylstilbestrol, 1,25 dihydroxy-vitamin D3, retinoic acid and ligand pharmaceutical analogs thereof (Mukherjee et al. *Nature*, 1997, 386: 407-410), dexmethasone, progesterone, thyroid hormone analogs (e.g. sodium liothyronine and sodium levothyronine), hexamethylene bisacetamide, 4-hydroxyquinazoline, coumarin and benzocaine.

Agents useful to increase the level of TGF-beta which is capable of binding to the TGF-beta receptors include agents that cause the release of TGF-beta from matrix components or plasma proteins, e.g., agents such as heparin sugar analogs and betaglycan proteoglycan chains, or cause the release of TGF-beta from lipoprotein complexes, e.g., agents such as vitamin E, simvastatin, VLDL-lowering agents, Apo-AII-lowering agents, and ApoAI-stimulating agents. Other agents useful to increase the level of TGF-beta which is capable of binding to the TGF-beta receptors include agents that cause an increase in the conversion of the latent form of TGF-beta to the active form of TGF-beta, e.g., hydrocortisone, dexmethasone, compounds of formula VI, vitamin D3, retinoic acid, simvastatin and thrombospondin.

Also provided is a kit comprising packing material enclosing, separately packaged, at least one device adapted for the delivery of a unit dosage form of a therapeutic agent and at least one unit dosage form comprising an amount of at least one of the therapeutic agents of the invention effective to accomplish at least one of the therapeutic results described herein when administered locally or systemically, as well as instruction means for its use, in accord with the present methods. As used herein, a "device adapted for delivery" of a therapeutic agent includes, but is not limited to, a catheter, a stent, a stent, a stent, a synthetic graft, and the like.

Also provided is a kit comprising packing material enclosing, separately packaged, at least one device adapted for the delivery of a therapeutic agent to a site in the lumen of a mammalian vessel and at least one unit dosage form of a first therapeutic agent and one unit dosage form of a second therapeutic agent effective to accomplish at least one of the therapeutic results described herein when administered locally or systemically, as well as instruction means for its use, in accord with the present methods.

Further provided is a pharmaceutical composition comprising a) at least one aspirinate, and b) at least one omega-3 fatty acid, wherein components (a) and (b) are present in a combined amount effective to maintain or increase TGF-beta levels, preferably at or near a site, or potential site, of atherosclerotic lesion formation or development.

The invention also provides a pharmaceutical composition comprising (a) an amount of a first agent effective to elevate the level of latent TGF-beta; and (b) an amount of a second agent effective to increase the level of TGF-beta which is capable of binding to the TGF-beta receptors.

The invention also provides a pharmaceutical composition comprising a) an aspirinate, such as copper 2-acetylacrylate or a compound of formula (I), and b) a compound of formula (VI), wherein components (a) and (b) are present in a combined amount effective to maintain or increase TGF-beta levels, preferably at or near a site, or potential site, of atherosclerotic lesion formation or development.

Also provided are novel compounds of formula (I), (II), (III), (IV), (V), (VI), (VII), or (VIII) or pharmaceutically acceptable salts thereof, and pharmaceutical compositions comprising a novel compound of formula (I), (II), (III), (IV), (V), (VI), (VII), or (VIII) as described herein or a pharmaceutically acceptable salt thereof, which are useful alone, or in combination, to elevate the level of TGF-beta in a mammal.

The invention also provides a therapeutic method. The method comprises identifying a patient exhibiting a decreased level of active TGF-beta and afflicted with a pathology associated with said decreased level. The patient so identified can be treated with an agent that elevates the levels of active TGF-beta so as to alleviate at least one of the symptoms of said pathology.

The invention also provides a method comprising determining endothelial cell activation in a mammal by detecting immunoglobulins that specifically bind to a TGF-beta Type II receptor or a portion thereof.

The invention also provides a method comprising diagnosing or monitoring a disease characterized by endothelial cell activation (e.g., atherosclerosis) in a mammal by detecting immunoglobulins that specifically bind to a TGF-beta Type II receptor or a portion thereof.

The invention also provides a method comprising detecting mammalian cells having TGF-beta Type II receptors, by combining the cells with a capture moiety that binds TGF-beta Type II receptors or a portion thereof, forming a capture complex, and detecting or determining the amount of the capture complex.

The invention also provides a kit comprising packaging material containing: a) a capture moiety comprising the extracellular domain of the TGF-beta Type II receptor; and b) a detection moiety capable of binding to an immunoglobulin. The invention also provides a kit comprising packaging material containing: a) a capture moiety that binds to the extracellular domain of the TGF-beta Type II receptor; and b) a detection moiety capable of binding to an immunoglobulin.

#### BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a schematic depicting the different forms of TGF-beta. TGF-beta is produced as a small latent complex (1) which is associated with the propeptide region termed LAP (thin black lines). During, or after secretion, of the small latent complex, additional proteins (hatched oval), e.g., LTBP-1, bind to the small latent complex to form the large latent complex (2). Latent complexes can be converted to the active form of TGF-beta, e.g., the 25 kD dimer (5) or the 25 kD dimer which is associated with a peptide of LAP (6). Examples of mature forms of TGF-beta are TGF-beta associated with lipoprotein (stippled oval) (3) or TGF-beta associated with a matrix protein (helical fiber) (4), e.g., fibrillin.

FIG. 2 depicts the association of increasing amounts of lipoprotein with (A) a reduction in TGF-beta binding to the TGF-beta receptor (R2X); and (B) an increasing amount of TGF-beta necessary to half maximally inhibit mink lung cell proliferation.

FIG. 3 depicts the association of TGF-beta with different lipoprotein classes. Profile of lipoprotein particle elution measured as total cholesterol (---) and TGF-beta elution (open circles) following separation of the lipoprotein fraction (d<1.215 g/cm<sup>3</sup>) by gel filtration chromatography. The position of the major lipoprotein classes are marked by reference to the elution times of the major apolipoproteins. (a) Healthy individual A (b) Healthy individual C (c) Dia-

betic individual K (d) Diabetic individual L. Letters designating the individuals shown refer to individuals in Table 1.

FIG. 4 depicts the effect of fish oil therapy on the association of TGF-beta with lipoprotein. Platelet-poor plasma was prepared from 36 individuals prior to receiving 5 g/day fish oil and then after 9 weeks with no fish oil supplementation.

FIG. 5 depicts the effect of aspirin on vascular smooth muscle cells. A) Dose response curve showing the inhibitory effects of increasing amounts of aspirin on human vascular smooth muscle cell proliferation. B) Percent increase in cell number in treated versus untreated human vascular smooth muscle cells.

FIG. 6 depicts the relationship between TGF-beta concentration found in the sera of normal individuals (A), individuals with triple vessel disease (B) and both populations (C), who were undergoing aspirin therapy.

FIG. 7 depicts the effect of tamoxifen (TMX) treatment on plasma TGF-beta over time. Active TGF-beta (●) and (a+1) TGF-beta (□) were assayed by ELISA in platelet poor plasma drawn at various times after beginning treatment with 40 mg/day TMX.

FIG. 8 depicts the effect of tamoxifen (TMX) on various cardiovascular risk factors. A) Lipoprotein(a) amounts. B) Proportion of TGF-beta associated with the lipoprotein fraction.

FIG. 9 depicts the lesion area in C57B 16, apo(a) or apo(E)- mice fed a normal diet, high fat diet or high fat diet supplemented with TMX.

FIG. 10 depicts the distribution of TGF-beta between the plasma (open segment) and various lipoprotein fractions at baseline (a) and after 8 hours during a fat tolerance test (b).

FIG. 11 shows the structure of the compounds MER25, zindoxifene, DDAC (Analog II), and DTAC (102b).

FIG. 12 depicts the pathways by which steroid and steroid-imide drugs act to produce anti-inflammatory effects and also undesirable side effects. The therapeutic action of ER/NFkB modulators is also depicted.

FIG. 13 depicts the pathway by which ER/NFkB modulators upregulate cellular mRNA encoding for TGF-beta.

#### DETAILED DESCRIPTION OF THE INVENTION

##### Administration of a Therapeutic Agent

The invention provides a method of treating a mammal having, or at risk of, a indication (e.g. a vascular indication) associated with a TGF-beta deficiency. The invention also provides a method to maintain elevated levels of TGF-beta in a mammal which is not imminently at risk of, or does not have, an indication associated with a deficiency in TGF-beta levels. The methods comprise the administration of at least one therapeutic agent that elevates the level of TGF-beta in said mammal. Preferably, the agent elevates the level of latent TGF-beta, for example by causing an increase in the level of TGF-beta mRNA, causing an increase in the translational efficiency of TGF-beta mRNA, or by causing an increase in the secretion of latent TGF-beta.

Another preferred embodiment is an agent that increases the level of TGF-beta which is capable of binding to the TGF-beta receptors, for example by causing the release of TGF-beta from matrix components of plasma proteins, by causing the release of TGF-beta from lipoprotein complexes, or by causing an increase in the conversion of the latent to the active form of TGF-beta.

Yet another embodiment of the invention employs the systemic administration of a therapeutic agent, e.g., a com-

pound of formula (I) including a pharmaceutically acceptable salt thereof, or a combination thereof, in an amount effective to inhibit or reduce the diminution in vessel lumen diameter in a diseased, e.g., atherosclerotic, or traumatized, e.g., due to stent placement, vessel.

Systemic administration of a therapeutic agent can also be employed to treat or prevent pre-atherosclerotic conditions, e.g., in patients at a high risk of developing atherosclerosis or exhibiting signs of hypertension resulting from atherosclerotic changes in vessels or vessel stenosis due to hypertrophy of the vessel wall. Preferably, the therapeutic agent is administered orally. It is also preferred that the agent useful in the practice of the invention is administered continually over a preselected period of time or administered in a series of spaced doses, i.e., intermittently, for a period of time as a preventative measure.

For the prevention of vessel lumen diminution associated with procedural vascular trauma, the therapeutic agent can be administered before, during or after the procedure, or any combination thereof. For example, for the prevention of restenosis, a series of spaced doses of the therapeutic agent, optionally, in sustained release dosage form, is preferably administered before, during and/or after the traumatic procedure (e.g., angioplasty). The dose may also be delivered locally, via a catheter introduced into the afflicted vessel during the procedure. After the traumatic procedure is conducted, a series of follow-up doses can be administered systemically over time, preferably in a sustained release dosage form, for a time sufficient to substantially reduce the risk of, or to prevent, restenosis. A preferred therapeutic protocol duration for this purpose involves administration from about 3 to about 26 weeks after angioplasty.

#### Combination Therapies

The invention provides combination therapies, i.e., the administration of at least two therapeutic agents which together are effective to maintain or elevate TGF-beta levels in a mammal. Accordingly, the invention provides a method of preventing or treating a mammal having, or at risk of, an indication which is associated with a TGF-beta deficiency, comprising administering an amount of a first agent effective to elevate the level of latent TGF-beta and an amount of a second agent effective to increase the level of TGF-beta which is capable of binding to the TGF-beta receptors, wherein said amounts are effective to increase the TGF-beta levels in said mammal.

The invention also provides a method comprising administering an amount of a combination of aspirin or an aspirinate and at least one omega-3 fatty acid, wherein said amount is effective to maintain or elevate the level of TGF-beta in said mammal.

The invention also provides a method of preventing or treating a mammal having, or at risk of, a vascular indication which is associated with a TGF-beta deficiency, comprising administering an effective amount of a combination of an aspirinate and at least one omega-3 fatty acid, wherein said amount is effective to increase the level of TGF-beta so as to inhibit or reduce vessel lumen diameter diminution. The invention also provides for the administration of at least two therapeutic agents which together are effective to elevate the levels of TGF-beta in a mammal so as to inhibit or reduce vessel lumen diameter diminution. The invention also provides combination therapies to maintain elevated levels of TGF-beta in a mammal which is not imminently at risk of, or does not have, a vascular indication associated with a deficiency in TGF-beta levels. The therapeutic agents can be selected to act in a synergistic, rather than in an additive, manner to elevate TGF-beta levels. The therapeutic agents

can be administered simultaneously as a single dose, simultaneously in individual doses, or sequentially.

One embodiment of the invention employs the systemic administration of a first therapeutic agent, e.g., an aspirinate such as copper 2-acetylsalicylate, a compound of formula (I), or a combination thereof, in combination with a second therapeutic agent, e.g., a compound of formula (VI), in an amount effective to increase TGF-beta levels. The increase in TGF-beta levels, in turn, inhibits or reduces the diminution in vessel lumen diameter in a diseased, e.g., atherosclerotic, or traumatized, e.g., due to stent placement, vessel. The increase in TGF-beta levels can also inhibit atherosclerotic lesion formation or development, increase plaque stability and/or promote lesion regression.

Systemic administration of the therapeutic agents can also be employed to treat or prevent pre-atherosclerotic conditions, e.g., in patients at a high risk of developing atherosclerosis or exhibiting signs of hypertension resulting from atherosclerotic changes in vessels or vessel stenosis due to hypertrophy of the vessel wall. Preferably, at least one of the therapeutic agents is administered orally.

It is also preferred that the agents useful in the practice of the invention are administered continually over a preselected period of time or administered in a series of spaced doses, i.e., intermittently, for a period of time as a preventative measure.

A preferred embodiment of the invention provides a method for the treatment or prevention of atherosclerosis, wherein an omega-3 fatty acid in combination with aspirin or an aspirinate, is administered so as to inhibit (block or reduce) atherosclerotic lesion formation or development, e.g., so as to inhibit lipid accumulation, increase plaque stability or promote lesion regression. In this embodiment of the invention, it is preferred that the therapeutic agents are orally administered. Preferably, copper aspirinate and an omega-3 fatty acid are orally administered. A preferred source of the omega-3 fatty acid is fish oil.

Another preferred embodiment of the invention provides a method for the treatment or prevention of atherosclerosis, wherein at least two therapeutic agents of the invention are administered in combination so as to inhibit (block or reduce) atherosclerotic lesion formation or development, e.g., so as to inhibit lipid accumulation, increase plaque stability or promote lesion regression. In this embodiment of the invention, it is preferred that at least one of the therapeutic agents is orally administered.

Combination therapies are also useful to treat vessels traumatized by interventional procedures. For example, for the prevention of restenosis, a series of spaced doses of at least two of the present therapeutic agents, optionally, in sustained release dosage form, are preferably administered before and after the traumatic procedure (e.g., angioplasty). The dose may also be delivered locally, via a catheter introduced into the afflicted vessel during the procedure. After the procedure is conducted, a series of follow-up doses of, optionally, both agents, can be administered systemically, preferably in a sustained release dosage form, for a time sufficient to substantially reduce the risk of, or to prevent, restenosis. As noted above, a preferred duration for this purpose is from about 3 to about 26 weeks after angioplasty.

Kits Comprising a Delivery Device and the Therapeutic Agents of the Invention

The invention provides a kit comprising packing material enclosing, separately packaged, at least one device adapted for the local or systemic delivery of a therapeutic agent, e.g., a catheter, a valve, a stent, a slit, a shunt or a synthetic graft, and at least one unit dosage form, as well as instruction

means for their use, in accord with the present methods. A valve, stent or shunt useful in the methods of the invention can comprise a biodegradable coating or porous non-biodegradable coating, having dispersed therein a therapeutic agent of the invention, preferably a sustained release dosage form of the therapeutic agent. The unit dosage form comprises an amount of at least one of the present therapeutic agents effective to accomplish the therapeutic results described herein when delivered locally and/or systemically. A preferred embodiment of the invention is a kit comprising a catheter adapted for the local delivery of at least one therapeutic agent to a site in the lumen of a mammalian vessel, along with instruction means directing its use in accord with the present invention. Preferably, the therapeutic agent comprises a copper aspirinate.

The invention provides a kit comprising packing material enclosing, separately packaged, at least one device adapted for the local or systemic delivery of a therapeutic agent, e.g., a catheter, a valve, a stent, a shunt or a synthetic graft, and at least one unit dosage form which may comprise an amount of at least two of the present therapeutic agents effective to accomplish the therapeutic results described herein.

Another embodiment of the invention is a kit comprising a catheter adapted for the local delivery of at least two therapeutic agents, a unit dosage of a first therapeutic agent, and a unit dosage of a second therapeutic agent, along with instruction means directing their use in accord with the present invention. The unit dosage forms of the first and second agents may be introduced via discrete lumens of a catheter, or mixed together prior to introduction into a single lumen of a catheter. If the unit dosage forms are introduced into discrete lumens of a catheter, the delivery of the agents to the vessel can occur simultaneously or sequentially. Moreover, a single lumen catheter may be employed to deliver a unit dosage form of one agent, followed by the reloading of the lumen with another agent and delivery of the other agent to the lumen of the vessel. Either or both unit dosages can act to reduce the diminution in vessel lumen diameter at the target site.

Alternatively, a unit dosage of one of the therapeutic agents may be administered locally, e.g., via catheter, while a unit dosage of another therapeutic agent is administered systemically, e.g., via oral administration. It is also envisioned that the kit of the invention comprises a non-catheter delivery device, e.g., a valve, stent, shunt or shunt, for systemic or local delivery of a compound of formula (I-VI). A valve, stent or shunt useful in the methods of the invention can comprise a biodegradable coating or porous non-biodegradable coating, having dispersed therein one or more therapeutic agents of the invention, preferably a sustained release dosage form of the therapeutic agent.

#### Definitions

The following definitions apply.

"Abnormal or pathological or inappropriate" with respect to an activity or proliferation means division, growth or migration of normal cells, but not cancerous or neoplastic cells, occurring more rapidly or to a significantly greater extent than typically occurs in a normally functioning cell of the same type, or in lesions not found in healthy tissues.

"Agents which activate the latent form of TGF-beta to the active form" include, but are not limited to, moieties such as hydrocortisone, dexamethasone, a compound of formula (VI) (such as tamoxifen), Vitamin D3 and retinoic acid (vitamin A); plasmin stimulators, e.g., Lp(a) lowering agents such as simvastatin, PAI-1 lowering agents (e.g., simvastatin and other VLDL-lowering agents), and agents which

exhibit increased tPA activity (e.g., retinoids, such as Vitamin D3); and agents which exhibit non-plasmin mediated activation (e.g., thrombospondin and Vitamin D3).

"Agents which increase the level of TGF-beta which is capable of binding to the TGF-beta receptors" includes moieties capable of activating the latent form of TGF-beta to the active form thereof, moieties which release TGF-beta from complexes of matrix components and TGF-beta, complexes of plasma proteins and TGF-beta and/or complexes of lipoproteins and TGF-beta. A number of compounds of formula (VI) can increase the level of TGF-beta which is capable of binding to the TGF-beta receptors.

"Agents which release TGF-beta from the extracellular matrix" include moieties such as heparin, heparin sugar analogs (e.g., fucoidin) and betaglycan proteoglycan chains.

"Agents which release TGF-beta from lipoprotein sequestration" include moieties such as Vitamin E and its salts (e.g., Vitamin E succinate), fish oil, simvastatin, other VLDL-lowering agents, apoAII-lowering agents, and apoAII-stimulating agents.

"ApoAII-lowering agent" includes an agent which decreases the synthesis of apoAII, decreases the post-translational insertion of apoAII into nascent HDL particles or stimulates the clearance of apoAII-containing particles, e.g., by immunopheresis of plasma with anti-apoAII antibodies.

"ApoAII-stimulating agent" includes an agent which stimulates the synthesis of apoAII, stimulates HDL production or extends the half-life of apoAII-HDL particles. For example, estrogen or estrogen agonists, or analogs and derivatives thereof, an agonist of hepatic nuclear factor (HNF) 3 or 4, or an agonist of the retinoid receptor, may increase apoAII transcription.

"Aspirinate" refers generally to aspirin derivatives and analogs, including pharmaceutically acceptable salts thereof, with the exception that aspirin itself is not included within the term "aspirinate". The term includes, but is not limited to, 3,5-diisopropyl salicylic acid, salicylic acid, 3,5-di(tertiarybutyl)salicylic acid, adamantylsalicylic acid, 3,5-dibromoacetylsalicylic acid, 3,5-diodoacetylsalicylic acid, 4-(tertiarybutyl)salicylic acid, 4-nitrosalicylic acid, 4-aminosalicylic acid, 4-acetylaminosalicylic acid, 5-chlorosalicylic acid, 3,5-dichlorosalicylic acid and salts thereof, and compounds of formula (I) and their salts. Preferably, the aspirinate is provided in essentially pure form, most preferably in a unit dosage form, in combination with one or more pharmaceutically acceptable carriers, including vehicles and/or excipients. Preferably, the aspirinate is in a form suitable for oral administration, and more preferably the aspirinate is in combination with a liquid vehicle.

"At least one", when used with respect to omega-3 fatty acids would be recognized in the art as indicating that a plurality, about 1 to 30, preferably about 1 to 25, more preferably about 2 to 20, of omega-3 fatty acids are often present in natural sources of these compounds.

"Autoimmune disease" means a disease which is characterized by the presence of autoreactive T lymphocytes resulting in pathological inflammation and subsequent damage or destruction of the target tissue. Such diseases include, but are not limited to, rheumatoid arthritis, multiple sclerosis and late-onset diabetes.

"Betaglycan proteoglycan chain" includes all or a portion of any of the proteoglycans that comprise the class of molecules termed type-III TGF-beta receptor, e.g., CD105, endoglin or betaglycan. For example, a portion of the proteoglycan may include all or a portion of the protein

moiety of the proteoglycan, all or a portion of the polysaccharide moiety of the proteoglycan, all or a portion of the protein moiety and a portion of the polysaccharide moiety, all or a portion of the polysaccharide moiety and a portion of the protein moiety, or a portion of the protein moiety and a portion of the polysaccharide moiety. Preferably, the betaglycan proteoglycan chain has a similar or greater affinity for TGF-beta relative to the affinity of native betaglycan for TGF-beta.

"Bioavailable" TGF-beta means TGF-beta which is in a form capable of binding to the TGF-beta receptors, i.e., eliciting a biological effect. For example, TGF-beta which is in a complex with matrix components or plasma proteins, or lipoproteins, is generally not "bioavailable" or has reduced bioavailability relative to TGF-beta which is not complexed with matrix components, plasma proteins, or lipoproteins.

"Cholesterol lowering agents" include agents which are useful for lowering serum cholesterol such as for example bile acid sequestering resins (e.g., colestipol hydrochloride or colestyramine), fibric acid derivatives (e.g., clofibrate, fenofibrate, or gemfibrozil), thiazolidenediones (e.g., troglitazone), or HMG-CoA reductase inhibitors (e.g., fluvastatin sodium, lovastatin, pravastatin sodium, or simvastatin), as well as nicotinic acid, niacin, or probucol.

"Elevated" TGF-beta levels means that the TGF-beta levels in vivo are greater after administration of the therapeutic agent than before administration. Thus, for example, active TGF-beta levels may be increased after administration, but may be less than normal levels, similar to normal levels or greater than normal levels of TGF-beta in vivo.

"Heparin sugar analogs" includes any sulfated polysaccharide which is a component of heparin sulfate proteoglycan, or a sulfated polysaccharide having a structure similar to the polysaccharide chain of heparin sulphate proteoglycan.

"NFkB" means any of the family of transcription factor complexes which have as at least one of their components the subunits known as p65 (RelA), p50, p52, c-rel, p68 (RelB) as well as the complexes which have as at least one of their components the endogenous inhibitors of NFkB activity, known as IkB-alpha, MAD3, p40, IkB-beta and IkB-gamma as well as their functional equivalents, analogs and derivatives thereof.

"NFkB activity" means activation of genes associated with the inflammatory state resulting from direct binding of an NFkB transcription factor complex to DNA elements, including, but not limited to, the kB element in the immunoglobulin kappa light chain gene. NFkB complex is normally retained in the cytoplasm by interaction with its endogenous inhibitor IkB. NFkB activity must be preceded by localization of the NFkB complex to the nucleus. However, translocation of the NFkB complex to the nucleus does not constitute NFkB activity unless transcription from genes associated with the inflammatory state is stimulated.

"Non-vascular indication" means diseases and conditions which are associated with TGF-beta deficiency, other than those diseases and conditions defined herein as vascular indications. Non-vascular indications include, but is not limited to cancer, Marfan's syndrome, Parkinson's disease, fibrosis, Alzheimer's disease, senile dementia, osteoporosis, diseases associated with inflammation, such as rheumatoid arthritis, multiple sclerosis and lupus erythematosus, as well as other auto-immune disorders. Non-vascular indications also include the promotion of wound healing and the lowering of serum cholesterol levels.

"Omega-3 fatty acid" includes synthetic or naturally occurring sources of omega-3 fatty acids, such as fish oil,

e.g., cod liver oil, walnuts and walnut oil, wheat germ oil, rapeseed oil, soybean lecithin, soybeans, tolu, common beans, butternuts, seaweed and flax seed oil. The omega-3 fatty acids include (C<sub>12</sub>-C<sub>22</sub>) alkanolic acids comprising 5-7 double bonds, wherein the last double bond is located between the third and fourth carbon atom from the methyl end of the fatty acid chain. These fatty acids have been proposed to yield significant cardiovascular protection (Burr et al., *Lancet*, 221, 757 (1989)). Omega-3 fatty acids include 5, 8, 11, 14, 17-tetracosapentaenoic acid and docosahexaenoic acid. See *The Merck Index* (11th ed. 1989) at entry 3495, and references cited therein.

"Pathological inflammation" means an increase in the recruitment and activation of immune cells, or residence and activation of immune cells for a longer period of time, in a particular tissue or tissues in an individual relative to an individual not at risk or, or afflicted with, an autoimmune disease. For the purposes of this description, the prototypical cells upon which the effects of ER/NFkB modulators are felt, are cells of the immune system, including but limited to, autoreactive T lymphocytes, alloreactive T lymphocytes, B lymphocytes, monocytes, tissue macrophages, neutrophils, eosinophils and other leukocytes. However, the usefulness of ER/NFkB modulators is not limited to their effects on immune cells in the treatment of autoimmune diseases. Effects on vascular endothelial cells and on the cells composing the target tissue may also contribute to the anti-inflammatory effect of the ER/NFkB modulators by reducing recruitment of leukocytes as well as activation of resident immune cells.

"PAI-1 lowering agent" includes an agent which increases insulin sensitivity, decreases production of PAI-1 or decreases the activity of PAI-1 as an inhibitor of plasminogen activators or of plasmin. PAI-1 lowering agent includes the thiazolidenediones (e.g., troglitazone).

"Plasmin stimulator" includes an agent which increases the activity of plasmin, e.g., a PAI-1 inhibitor, tissue plasminogen activator (tPA) or streptokinase, preferably without disrupting normal hemostasis. A plasmin stimulator may increase plasmin levels by catalyzing the conversion of the latent form of plasmin, i.e., plasminogen, to the active form, or stimulate the activity of the plasmin enzyme, e.g., generally or with regard to a specific substrate, e.g., TGF-beta.

"Procedural vascular trauma" includes the effects of surgical/medical-mechanical interventions into mammalian vasculature, but does not include vascular trauma due to the organic vascular pathologies listed hereinabove, or to unintended traumas, such as due to an accident. Thus, procedural vascular traumas within the scope of the present treatment method include (1) organ grafting or transplantation, such as transplantation and grafting of heart, kidney, liver and the like, e.g., involving vessel anastomosis; (2) vascular surgery, such as coronary bypass surgery, biopsy, heart valve replacement, atherectomy, thrombectomy, and the like; (3) transcatheter vascular therapies (TVT) including angioplasty, e.g., laser angioplasty and PTCA procedures discussed hereinbelow, employing balloon catheters, or indwelling catheters; (4) vascular grafting using natural or synthetic materials, such as in saphenous vein coronary bypass grafts, dacron and venous grafts used for peripheral arterial reconstruction, etc.; (5) placement of a mechanical shunt, such as a PTFE hemodialysis shunt used for arteriovenous communications; and (6) placement of an intravascular stent, which may be metallic, plastic or a biodegradable polymer. See U.S. patent application Ser. No. 08/389,712, filed Feb. 15, 1995, which is incorporated by reference herein. For a general discussion of implantable devices and

biomaterials from which they can be formed, see H. Kambic et al., "Biomaterials in Artificial Organs", *Chem. Eng. News*, 30 (Apr. 14, 1986), the disclosure of which is incorporated by reference herein.

"Proliferation," means an increase in cell number, i.e., by mitosis of the cells.

"Sustained release" means a dosage form designed to release a therapeutic agent therefrom for a time period ranging from at least about 0.0005 to about 21, and more preferably at least about 1-3 to about 120, days. Release over a longer time period is also contemplated as "sustained release" in the context of the dosage form of the present invention. It is contemplated that sustained release dosage forms for systemic administration as well as local administration can be employed in the practice of the invention. Examples of sustained release dosage forms are disclosed in co-pending application Ser. No. 08/478,936, filed Jun. 7, 1995, the disclosure of which is incorporated by reference herein.

"Tamoxifen" includes trans-2-[4-[(1,2-diphenyl-1-butenyl)phenoxy]-N,N-dimethylethylamine, and the pharmaceutically acceptable salts thereof, which are capable of enhancing the level of active TGF- $\beta$ , e.g., by increasing the level of latent TGF- $\beta$  or by increasing the level of TGF- $\beta$  which is capable of binding to the TGF- $\beta$  receptors.

"TGF- $\beta$ " includes transforming growth factor- $\beta$ s as well as functional equivalents, derivatives and analogs thereof, e.g., TGF- $\beta_1$ , TGF- $\beta_2$ , and TGF- $\beta_3$ . The TGF- $\beta$  isoforms are a family of multifunctional, disulfide-linked dimeric polypeptides that affect activity, proliferation and differentiation of various cells types. A functional equivalent of TGF- $\beta$  can include agents that bind to the TGF- $\beta$  receptor, e.g., a receptor agonist or antagonist or a neutral binding agent, and/or which induces the same biological response as TGF- $\beta$ .

"Vascular indication" includes, but is not limited to, a cardiovascular disease, e.g., atherosclerosis, thrombosis, myocardial infarction, and stroke, or a cardiovascular condition, e.g., vessels subjected to trauma associated with interventional procedures ("procedural vascular trauma"), such as stenosis following angioplasty, placement of a shunt, stent, stenosis or natural excision grafts, indwelling catheter, valve or other implantable devices. Also within the scope of the term "vascular indication" is non-coronary vessel disease, such as arteriosclerosis, small vessel disease, nephropathy, greater than normal levels of serum cholesterol, asthma, hypertension, emphysema and chronic obstructive pulmonary disease. "Vascular indication" does not include cancer, including smooth muscle cell carcinomas or neoplasms, or idiopathic symptoms such as forms of angina that are not attributable to vascular diseases.

Small vessel disease includes, but is not limited to, vascular insufficiency in the limbs, peripheral neuropathy and retinopathy, e.g., diabetic retinopathy. "VLDL-lowering agent" includes an agent which decreases the hepatic synthesis of triglyceride-rich lipoproteins or increases the catabolism of triglyceride-rich lipoproteins, e.g., fibrates such as gemfibrozil, or the statins, increases the expression of the apoE-mediated clearance pathway, or improves insulin sensitivity in diabetics, e.g., the thiazolidine diones.

Additionally, as used herein, "agents which increase the level of latent TGF- $\beta$ " include moieties capable of stimulating the production of TGF- $\beta$  protein (generally the latent form thereof). The mechanism leading to the increase in TGF- $\beta$  protein can include, but is not limited to, up-regulation of mRNA production (transcription),

increased translational efficiency of the mRNA, or increased secretion of the latent TGF- $\beta$  complex. Agents which increase the production of TGF- $\beta$  protein include, but are not limited to, moieties which affect the nuclear hormone receptor pathway (e.g., tamoxifen, idoxifen, toremifene, raloxifene, toloxifen, and other anti-estrogen analogues of tamoxifen, ethynyl estradiol, diethylstilbestrol, other synthetic estrogen agonists and compounds disclosed in U.S. Pat. Nos. 4,442,119, 5,015,666, 5,098,903, 5,324,736), 1,25 dihydroxy-vitamin D<sub>3</sub>, allopurinol, EG 1089, MC<sub>203</sub>, KHI060, retinoic acid/vitamin A and ligand pharmaceutical analogs thereof (Mukherjee et al. *Nature*, 1997, 386: 407-410), dexamethasone (e.g., glucocorticoid agonist analogues), progesterone (e.g., gestodene and synthetic progestins), and thyroid hormone analogues (e.g., sodium liothyronine and sodium levothyroxine), (e.g., 12,14 didioxy-prostaglandin J<sub>2</sub>, A12,14-PGJ<sub>2</sub>).

Other agents which increase the level of TGF- $\beta$  include aspirin, aspirinates such as copper aspirinate, and red wine extract (see Example IV). Red wine extract is a fraction or concentrate derived from red wine that is substantially enriched in copper aspirinate, hexamethylene bisacetamide, 4-hydroxyquinazoline, coumarin and benzocaine.

The term "halo" includes fluoro, chloro, bromo, or iodo. The terms alkyl, and alkoxy denote both straight and branched groups; but reference to an individual radical such as "propyl" embraces only the straight chain radical, a branched chain isomer such as "isopropyl" being specifically referred to. Aryl denotes a phenyl radical or an ortho-fused bicyclic carbocyclic radical having about nine to ten ring atoms in which at least one ring is aromatic. Heteroaryl encompasses a radical attached via a ring carbon of a monocyclic aromatic ring containing five or six ring atoms consisting of carbon and one to four heteroatoms each selected from the group consisting of non-peroxide oxygen, sulfur, and N(X) wherein X is absent or is hydrogen, O, (C<sub>1</sub>-C<sub>2</sub>)alkyl, phenyl or benzyl, as well as a radical of an ortho-fused bicyclic heterocycle of about eight to ten ring atoms derived therefrom, particularly a benz-derivative or one derived by fusing a propylene, trimethylene, or tetramethylene diradical thereto.

It will be appreciated by those skilled in the art that compounds of the invention having a chiral center may exist in and be isolated in optically active and racemic forms. Some compounds may exhibit polymorphism. It is to be understood that the present invention encompasses any racemic, optically-active, polymorphic, or stereoisomeric form, or mixtures thereof, of a compound of the invention, which possess the useful properties described herein, it being well known in the art how to prepare optically active forms (for example, by resolution of the racemic form by recrystallization techniques, by synthesis from optically-active starting materials, by chiral synthesis, or by chromatographic separation using a chiral stationary phase).

Specific values listed for radicals, substituents, and ranges, are for illustration only and they do not exclude other defined values or other values within defined ranges for the radicals and substituents.

Specifically, (C<sub>1</sub>-C<sub>2</sub>)alkyl can be methyl, ethyl, propyl, or isopropyl; (C<sub>1</sub>-C<sub>3</sub>)alkyl can be methyl, ethyl, propyl, isopropyl, butyl, iso-butyl or sec-butyl; (C<sub>1</sub>-C<sub>4</sub>)alkyl can be methyl, ethyl, propyl, isopropyl, butyl, iso-butyl, sec-butyl, pentyl, isopentyl, neo-pentyl, hexyl, 2-hexyl, 3-hexyl, heptyl, 2-heptyl, 3-heptyl, octyl, 2-octyl, 3-octyl, 4-octyl, nonyl, 2-nonyl, 3-nonyl, 4-nonyl, decyl, 2-decyl, 3-decyl,

4-decyl, 5-decyl, undecyl, 2-undecyl, 3-undecyl, 4-undecyl, 5-undecyl, dodecyl, 2-dodecyl, 3-dodecyl, 4-dodecyl, 5-dodecyl, or 6-dodecyl; (C<sub>7</sub>-C<sub>8</sub>)cycloalkyl can be cyclopropyl, cyclobutyl, cyclopentyl, or cyclohexyl; (C<sub>7</sub>-C<sub>8</sub>)cycloalkenyl can be cyclopropenyl, cyclobutenyl, cyclopentenyl, cyclopentadienyl, cyclohexenyl, or cyclohexadienyl; (C<sub>1</sub>-C<sub>2</sub>)alkoxy can be methoxy, ethoxy, propoxy, isopropoxy, butoxy, iso-butoxy, sec-butoxy, pentyloxy, neopentyloxy, isopentyloxy, or hexyloxy; (C<sub>1</sub>-C<sub>6</sub>)alkanoyl can be acetyl, propanoyl or butanoyl; (C<sub>7</sub>-C<sub>8</sub>)alkanoyloxy can be acetoxy, propanoyloxy or butanoyloxy; halo(C<sub>1</sub>-C<sub>12</sub>)alkyl can be fluoromethyl, difluoromethyl, trifluoromethyl, fluoroethyl, difluoroethyl, trifluoroethyl, perfluoroethyl, fluoroisopropyl, difluoroisopropyl, trifluoroisopropyl, fluoroisobutyl, difluoroisobutyl, trifluoroisobutyl, fluoroisopentyl, difluoroisopentyl, trifluoroisopentyl, fluoroisohexyl, difluoroisohexyl, trifluoroisohexyl, chloroethyl, dichloroethyl, trichloroethyl, trifluoroethyl, chloropropyl, dichloropropyl, trichloropropyl, chlorobutyl, dichlorobutyl, trichlorobutyl, chloropentyl, dichloropentyl, trichloropentyl, chlorohexyl, dichlorohexyl, trichlorohexyl, bromoethyl, dibromoethyl, tribromoethyl, perbromoethyl, bromopropyl, dibromopropyl, tribromopropyl, bromobutyl, dibromobutyl, tribromobutyl, bromopentyl, dibromopentyl, tribromopentyl, bromohexyl, dibromohexyl, tribromohexyl, iodoethyl, iodopropyl, iodobutyl, iodopentyl, iodohexyl, haloheptyl, dihaloheptyl, trihaloheptyl, halooctyl, dihalooctyl, trihalooctyl, halononyl, dihalononyl, trihalononyl, haloodecyl, dihalodecyl, trihalodecyl, haloundecyl, dihaloundecyl, trihaloundecyl, halododecyl, dihalododecyl, or trihalododecyl.

Likewise, aryl can be phenyl, indenyl, or naphthyl; heteroaryl can be furyl, imidazolyl, tetrazolyl, pyridyl, (or its N-oxide), thienyl, pyrimidinyl (or its N-oxide), indolyl, or quinolyl (or its N-oxide); and aryl (C<sub>1</sub>-C<sub>3</sub>)alkyl can be benzyl, indenylmethyl, naphthylmethyl, phenethyl, indenylethyl, naphthylethyl, phenylpropyl, indenylpropyl, or naphthylpropyl; and heteroaryl (C<sub>1</sub>-C<sub>3</sub>)alkyl can be furylmethyl, imidazolylmethyl, tetrazolylmethyl, pyridylmethyl (or its N-oxide), thienylmethyl, pyrimidinylmethyl (or its N-oxide), indolylmethyl, quinolylmethyl, furylethyl, imidazolethyl, tetrazolethyl, pyridylethyl, (or its N-oxide), thienylethyl, pyrimidinylethyl (or its N-oxide), indolylethyl, quinolylethyl, furylpropyl, imidazolepropyl, tetrazolepropyl, pyridylpropyl, (or its N-oxide), thienylpropyl, pyrimidinylpropyl (or its N-oxide), indolylpropyl, or quinolylpropyl.

More specifically, (C<sub>1</sub>-C<sub>3</sub>)alkyl can be methyl, ethyl, or propyl; (C<sub>1</sub>-C<sub>6</sub>)alkyl can be methyl, ethyl, propyl, or butyl; (C<sub>1</sub>-C<sub>8</sub>)alkyl can be methyl, ethyl, propyl, isopropyl, butyl, iso-butyl, pentyl, or hexyl; (C<sub>1</sub>-C<sub>12</sub>)alkyl can be methyl, ethyl, propyl, isopropyl, butyl, iso-butyl, sec-butyl, pentyl, hexyl, heptyl, or octyl; (C<sub>7</sub>-C<sub>8</sub>)cycloalkyl can be cyclopropyl, or cyclohexyl; (C<sub>7</sub>-C<sub>8</sub>)cycloalkenyl can be 2-cyclopropenyl, 3-cyclopentenyl, 2-cyclohexenyl, or 3-cyclohexenyl; (C<sub>1</sub>-C<sub>6</sub>)alkoxy can be methoxy, ethoxy, or propoxy; (C<sub>1</sub>-C<sub>6</sub>)alkanoyl can be acetyl; (C<sub>7</sub>-C<sub>8</sub>)alkanoyloxy can be acetoxy; halo(C<sub>1</sub>-C<sub>12</sub>)alkyl can be fluoromethyl, difluoromethyl, trifluoromethyl, trifluoroethyl, fluoroisopropyl, trifluoroisopropyl, fluoroisobutyl, trifluoroisobutyl, trifluoroisopentyl, fluoroisohexyl, trifluoroisohexyl, chloroethyl, chloropropyl, chlorobutyl, bromoethyl, bromopropyl, bromobutyl, iodoethyl, iodopropyl, iodobutyl; aryl can be phenyl, heteroaryl can be furyl, imidazolyl, pyridyl (or its N-oxide), or thienyl; aryl (C<sub>1</sub>-C<sub>3</sub>)alkyl can be benzyl or phenethyl, and heteroaryl (C<sub>1</sub>-C<sub>3</sub>)alkyl can be furylmethyl, imidazolylmethyl, pyridylmethyl (or its N-oxide), or thienylmethyl.

Compounds of Formula (I) Within the Scope of the Invention

A specific aspirinate useful in the present invention is a compound of formula (I):



wherein

R<sup>1</sup> is hydrogen, halo, nitro, cyano, hydroxy, CF<sub>3</sub>, —NR<sub>6</sub>R<sub>6</sub>, —C(=O)OR<sub>6</sub>, —OC(O)OR<sub>6</sub>, —C(=N)OR<sub>6</sub>, (C<sub>1</sub>-C<sub>3</sub>)alkyl or (C<sub>1</sub>-C<sub>6</sub>)alkoxy;

R<sup>2</sup> is hydrogen or —XR<sub>6</sub>;

R<sup>3</sup> is —C(=O)YR<sub>6</sub>;

R<sup>4</sup> is (=O)<sub>2</sub>; or R is (C<sub>1</sub>-C<sub>3</sub>)alkyl, (C<sub>1</sub>-C<sub>6</sub>) alkanoyl or (C<sub>7</sub>-C<sub>8</sub>)alkanoyloxy and forms a sulfonium salt with the thiophene sulfur, wherein the associated counter ion is a pharmaceutically acceptable anion;

R<sup>5</sup> is hydrogen;

n is 0, 1 or 2;

X is oxygen or sulfur;

Y is oxygen or sulfur;

R<sub>6</sub> is (C<sub>1</sub>-C<sub>3</sub>)alkanoxy;

R<sub>6</sub> is hydrogen or (C<sub>1</sub>-C<sub>3</sub>)alkyl;

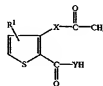
R<sub>6</sub> and R<sub>6</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>3</sub>) alkyl, phenyl, C(=O)OH, C(=O)O(C<sub>1</sub>-C<sub>3</sub>)alkyl, CH<sub>2</sub>C(=O)OH, CH<sub>2</sub>C(=O)O(C<sub>1</sub>-C<sub>3</sub>)alkyl, or (C<sub>1</sub>-C<sub>6</sub>)alkoxy; or R<sub>6</sub> and R<sub>6</sub> together with the nitrogen to which they are attached are a 3, 4, 5, or 6 membered heterocyclic ring; and

R<sub>6</sub> is hydrogen or (C<sub>1</sub>-C<sub>3</sub>)alkyl;

or a pharmaceutically acceptable salt thereof;

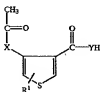
provided that R<sup>2</sup> and R<sup>3</sup> are on adjacent positions of the ring to which they are attached, or are on the 2- and 5-positions of the ring; and further provided that when R<sup>2</sup> is hydrogen; R<sup>3</sup> is on the 2- or 5-position of the ring to which it is attached and R<sup>4</sup> is (C<sub>1</sub>-C<sub>6</sub>)alkanoyloxy.

A specific aspirinate of formula I useful in the present invention is a compound of formula (II):



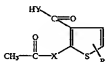
wherein X is O or S; Y is O or S; R<sup>1</sup> is hydrogen, halo, nitro, cyano, hydroxy, CF<sub>3</sub>, —NR<sub>6</sub>R<sub>6</sub>, —C(=O)OR<sub>6</sub>, —OC(=O)OR<sub>6</sub>, —C(=N)OR<sub>6</sub>, (C<sub>1</sub>-C<sub>3</sub>)alkyl or (C<sub>1</sub>-C<sub>6</sub>)alkoxy; and R<sub>6</sub> and R<sub>6</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>3</sub>)alkyl, phenyl, —C(=O)OH, —C(=O)O(C<sub>1</sub>-C<sub>3</sub>)alkyl, —CH<sub>2</sub>C(=O)OH, —CH<sub>2</sub>C(=O)O(C<sub>1</sub>-C<sub>3</sub>)alkyl, or (C<sub>1</sub>-C<sub>6</sub>)alkoxy; or R<sub>6</sub> and R<sub>6</sub> together with the nitrogen to which they are attached are a 3, 4, 5, or 6 membered heterocyclic ring; or a pharmaceutically acceptable salt thereof

A specific aspirinate of formula I useful in the present invention is a compound of formula (III):



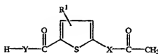
wherein X is O or S; Y is O or S; R<sup>1</sup> is hydrogen, halo, nitro, cyano, hydroxy, CF<sub>3</sub>, —NR<sub>2</sub>, —C(=O)OR<sub>2</sub>, —OC(=O)OR<sub>2</sub>, —C(=N)OR<sub>2</sub>, (C<sub>1</sub>-C<sub>6</sub>)alkyl or (C<sub>1</sub>-C<sub>6</sub>)alkoxy; R<sub>2</sub> and R<sub>3</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, phenyl, —C(=O)OH, —C(=O)O(C<sub>1</sub>-C<sub>6</sub>)alkyl, —CH<sub>2</sub>C(=O)OH, —CH<sub>2</sub>C(=O)O(C<sub>1</sub>-C<sub>6</sub>)alkyl, or (C<sub>1</sub>-C<sub>6</sub>)alkoxy; or R<sub>2</sub> and R<sub>3</sub> together with the nitrogen to which they are attached are a 3, 4, 5, or 6 membered heterocyclic ring; or a pharmaceutically acceptable salt thereof.

Another specific aspirinate of formula I useful in the present invention is a compound of formula (IV):



wherein X is O or S; Y is O or S; R<sup>1</sup> is hydrogen, halo, nitro, cyano, hydroxy, CF<sub>3</sub>, —NR<sub>2</sub>, —C(=O)OR<sub>2</sub>, —OC(=O)OR<sub>2</sub>, —C(=N)OR<sub>2</sub>, (C<sub>1</sub>-C<sub>6</sub>)alkyl or (C<sub>1</sub>-C<sub>6</sub>)alkoxy; R<sub>2</sub> and R<sub>3</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, phenyl, —C(=O)OH, —C(=O)O(C<sub>1</sub>-C<sub>6</sub>)alkyl, —CH<sub>2</sub>C(=O)OH, —CH<sub>2</sub>C(=O)O(C<sub>1</sub>-C<sub>6</sub>)alkyl, or (C<sub>1</sub>-C<sub>6</sub>)alkoxy; or R<sub>2</sub> and R<sub>3</sub> together with the nitrogen to which they are attached are a 3, 4, 5, or 6 membered heterocyclic ring; or a pharmaceutically acceptable salt thereof.

Another specific aspirinate of formula I useful in the present invention is a compound of formula (V):



wherein X is O or S; Y is O or S; R<sup>1</sup> is hydrogen, nitro, halo, cyano, hydroxy, or N(R<sub>2</sub>), wherein each R<sub>2</sub> is hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, phenyl, COOH, CO<sub>2</sub>(C<sub>1</sub>-C<sub>6</sub>)alkyl, or O[(C<sub>1</sub>-C<sub>6</sub>)alkyl]; R<sub>2</sub> and R<sub>3</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, phenyl, —C(=O)OH, —C(=O)O(C<sub>1</sub>-C<sub>6</sub>)alkyl, —CH<sub>2</sub>C(=O)OH, —CH<sub>2</sub>C(=O)O(C<sub>1</sub>-C<sub>6</sub>)alkyl, or (C<sub>1</sub>-C<sub>6</sub>)alkoxy; or R<sub>2</sub> and R<sub>3</sub> together with the nitrogen to which they are attached are a 3, 4, 5, or 6 membered heterocyclic ring; or a pharmaceutically acceptable salt thereof.

A specific aspirinate useful in the present invention is a compound of formula (I):



wherein

R<sup>1</sup> is hydrogen, halo, nitro, cyano, hydroxy, CF<sub>3</sub>, —NR<sub>2</sub>, —C(=O)OR<sub>2</sub>, (C<sub>1</sub>-C<sub>6</sub>)alkyl or (C<sub>1</sub>-C<sub>6</sub>)alkoxy;

R<sup>2</sup> is hydrogen or —XR<sub>3</sub>;

R<sup>3</sup> is —C(=O)YR<sub>4</sub>;

R<sup>4</sup> is (—O)<sub>n</sub>; or R<sup>4</sup> is (C<sub>1</sub>-C<sub>6</sub>)alkyl, (C<sub>1</sub>-C<sub>6</sub>)alkanoyl or (C<sub>1</sub>-C<sub>6</sub>)alkanoyloxy and forms a sulfonium salt with the thiophene sulfur, wherein the associated counter ion is a pharmaceutically acceptable anion;

R<sup>5</sup> is hydrogen;

n is 0, 1 or 2;

X is oxygen or sulfur;

Y is oxygen or sulfur;

R<sub>2</sub> is (C<sub>1</sub>-C<sub>6</sub>)alkanoyl;

R<sub>3</sub> is hydrogen or (C<sub>1</sub>-C<sub>6</sub>)alkyl;

R<sub>4</sub> and R<sub>5</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, phenyl, COOH, CO<sub>2</sub>(C<sub>1</sub>-C<sub>6</sub>)alkyl or O[(C<sub>1</sub>-C<sub>6</sub>)alkyl]; or R<sub>4</sub> and R<sub>5</sub> together with the nitrogen to which they are attached are pyrrolidino, piperidino, piperazino, 1-ly or morpholino; and

R<sub>5</sub> is hydrogen or (C<sub>1</sub>-C<sub>6</sub>)alkyl;

or a pharmaceutically acceptable salt thereof;

provided that R<sup>2</sup> and R<sup>3</sup> are on adjacent positions of the ring to which they are attached, or are on the 2- and 5-positions of the ring; and further provided that when R<sup>2</sup> is hydrogen; R<sup>3</sup> is on the 2- or 5-position of the ring to which it is attached and R<sup>4</sup> is (C<sub>1</sub>-C<sub>6</sub>)alkanoyloxy.

A specific aspirinate useful in the present invention is a compound of formula (I) which is not 3-acetoxy-2-carboxythiophene.

Another specific aspirinate useful in the present invention is a compound of formula (I) wherein R<sup>1</sup> is halo, nitro, cyano, CF<sub>3</sub> or —C(=O)OR<sub>2</sub>; or a pharmaceutically acceptable salt thereof.

Yet another specific aspirinate useful in the present invention is a compound of formula (I) wherein R<sup>1</sup> is hydrogen.

A further specific aspirinate useful in the present invention is a compound of formula (I) wherein R<sup>2</sup> is —XR<sub>3</sub>.

A specific aspirinate useful in the present invention is a compound of formula (I) wherein R<sup>4</sup> is (C<sub>1</sub>-C<sub>6</sub>)alkanoyl or (C<sub>1</sub>-C<sub>6</sub>)alkanoyloxy and forms a sulfonium salt with the thiophene sulfur, wherein the associated counter ion is a pharmaceutically acceptable anion.

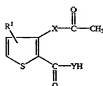
A specific aspirinate useful in the present invention is a compound of formula (I) wherein R<sup>5</sup> is hydrogen.

Another specific aspirinate useful in the present invention is a compound of formula (I) wherein R<sup>2</sup> is in the 3-position, R<sup>3</sup> is in the 4-position and R<sup>4</sup> is halo, nitro, cyano, hydroxy, CF<sub>3</sub>, —NR<sub>2</sub>, —C(=O)OR<sub>2</sub>, (C<sub>1</sub>-C<sub>6</sub>)alkyl, or (C<sub>1</sub>-C<sub>6</sub>)alkoxy; or a pharmaceutically acceptable salt thereof.

Yet another specific aspirinate useful in the present invention is a compound of formula (I) wherein R<sup>2</sup> is in the

2-position and R<sup>3</sup> is in the 3-position; and R<sup>1</sup> is halo, nitro, cyano, hydroxy, CF<sub>3</sub>, —NR<sup>4</sup>, —C(=O)OR<sup>4</sup>, (C<sub>1</sub>—C<sub>6</sub>) alkyl, or (C<sub>1</sub>—C<sub>6</sub>)alkoxy; or a pharmaceutically acceptable salt thereof.

A specific aspirinate of formula I useful in the present invention is a compound of formula (II):



wherein X is O or S; Y is O or S; R<sup>1</sup> is hydrogen, nitro, halo, cyano, hydroxy, or N(R)<sub>2</sub>, wherein each R is hydrogen, (C<sub>1</sub>—C<sub>6</sub>)alkyl, phenyl, COOH, CO<sub>2</sub>(C<sub>1</sub>—C<sub>6</sub>)alkyl, or O[(C<sub>1</sub>—C<sub>6</sub>)alkyl], or a pharmaceutically acceptable salt thereof.

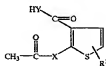
A specific aspirinate of formula I useful in the present invention is a compound of formula II wherein, if X=Y=O, then R<sup>1</sup> is not H.

A specific aspirinate of formula I useful in the present invention is a compound of formula (III):



wherein X is O or S; Y is O or S; R<sup>1</sup> is hydrogen, nitro, halo, cyano, hydroxy, or N(R)<sub>2</sub>, wherein each R is hydrogen, (C<sub>1</sub>—C<sub>6</sub>)alkyl, phenyl, COOH, CO<sub>2</sub>(C<sub>1</sub>—C<sub>6</sub>)alkyl, or O[(C<sub>1</sub>—C<sub>6</sub>)alkyl]; or a pharmaceutically acceptable salt thereof.

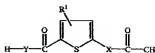
Another specific aspirinate of formula I useful in the present invention is a compound of formula (IV):



wherein X is O or S; Y is O or S; R<sup>1</sup> is hydrogen, nitro, halo, cyano, hydroxy, or N(R)<sub>2</sub>, wherein each R is hydrogen, (C<sub>1</sub>—C<sub>6</sub>)alkyl, phenyl, COOH, CO<sub>2</sub>(C<sub>1</sub>—C<sub>6</sub>)alkyl, or O[(C<sub>1</sub>—C<sub>6</sub>)alkyl], or a pharmaceutically acceptable salt thereof.

Another specific aspirinate of formula I useful in the present invention is a compound of formula (V):

(V)



wherein X is O or S; Y is O or S; R<sup>1</sup> is hydrogen, nitro, halo, cyano, hydroxy, or N(R)<sub>2</sub>, wherein each R is hydrogen, (C<sub>1</sub>—C<sub>6</sub>)alkyl, phenyl, COOH, CO<sub>2</sub>(C<sub>1</sub>—C<sub>6</sub>)alkyl, or O[(C<sub>1</sub>—C<sub>6</sub>)alkyl], or a pharmaceutically acceptable salt thereof.

Another specific aspirinate useful in the present invention is a compound of formula II, III, IV or V wherein R<sup>1</sup> is hydrogen; or a pharmaceutically acceptable salt thereof.

Another specific aspirinate useful in the present invention is a compound of formula II, III, IV or V wherein R<sup>1</sup> is nitro, halo, cyano, hydroxy, or N(R)<sub>2</sub>, wherein each R is hydrogen, (C<sub>1</sub>—C<sub>6</sub>)alkyl, phenyl, COOH, CO<sub>2</sub>(C<sub>1</sub>—C<sub>6</sub>)alkyl, or O[(C<sub>1</sub>—C<sub>6</sub>)alkyl], or a pharmaceutically acceptable salt thereof.

Another specific aspirinate useful in the present invention is a compound of formula II, III, IV or V wherein X is S.

Another specific aspirinate useful in the present invention is a compound of formula II, III, IV or V wherein Y is S.

The compounds of formulas (I), like aspirin, can transfer acetyl functionality. Moreover, the thiophene ring skeleton of compounds of formulas (I), which is similar in size to the benzene ring system in aspirin, results in a similar biodistribution, pharmacokinetics and pharmacodynamics for these compounds relative to aspirin. Furthermore, the thiophene ring sulfur (n=0) in a compound of formula I can be readily catabolized to sulfone (n=2) and sulfoxide (n=1), which increases the water solubility of the compounds, so they can be rapidly excreted. This rapid catabolism reduces the gastric irritation, gastric ulcers and occasional bleeding observed with high doses of aspirin, as well as kidney retention leading to crystal urea and kidney stones, all of which are due to the insolubility of salicylates and divalent and trivalent complexes of salicylates with metals. Besides being useful as TGF- $\beta$  elevating agents, the compounds of formula (I) are useful as anti-inflammatory agents, e.g., as anti-platelet aggregation agents, thrombin inhibitory agents, and vascular smooth muscle cell anti-proliferative agents.

Furthermore, substitution of electron withdrawing and electron donating functionalities on the thiophene ring system can enhance or diminish the bioavailability of the substituted compounds. Thus, some of the substituted compounds exhibit higher protein binding affinities, and thus have higher binding affinities to serum proteins. The higher binding affinities lead to a longer serum half-life, which provides a longer duration of action for the compounds. Other substituted compounds exhibit lower protein binding affinities, and thus have lower binding affinities to serum proteins. The lower binding affinities lead to a shorter serum half-life, which provides a shorter duration of action for the compounds. Moreover, the compounds of formula (I) can chelate metal ions, which can result in enhanced transport across membranes.

The aspirinates of the invention preferably include copper salts, as well as alkali metal or alkaline earth metal aspirinate salts, such as lithium, sodium, potassium, magnesium, zinc, or calcium aspirinate salts, although other salts are envisioned.

The copper aspirinate salts of the invention can be formed for example by reacting a copper salt such as cupric chloride

with the sodium salts of 3,5-diisopropyl salicylic acid, acetylsalicylic acid, salicylic acid, 3,5-di-tert-butyl salicylic acid, adamantylsalicylic acid, 3,5-dibromoacetylsalicylic acid, 3,5-diiodoacetylsalicylic acid, 4-tert-butylsalicylic acid, 4-nitrosalicylic acid, 4-aminosalicylic acid, 4-acetylaminosalicylic acid, 5-chlorosalicylic acid and 3,5-dichlorosalicylic acid.

The copper salt of a thiophene-ring based analog or derivative of an aspirinate of the invention can be prepared by reacting a copper salt, e.g., cupric chloride, with the sodium salt of the thiophene based analog or derivative.

Inorganic copper salts useful in synthesizing copper aspirinate salts of the invention include hydrated copper chloride, and the hydrate thereof, copper fluorosilicate and the hydrate thereof, copper sulfate and the pentahydrate thereof, copper nitrate and the tri- and hexa-hydrates thereof, copper bromide, copper metaborate, copper bromate, copper chlorate, copper iodate and copper fluorophosphate. In the above salts, the copper is typically in the Copper (II) oxidation state.

It is preferable to produce copper aspirinate coordination solvates rather than anhydrous compounds. The copper aspirinate compounds may be solvated with a lower alcohol, e.g., a  $C_2-C_6$  aliphatic alcohol such as ethanol or isopropanol, a ketone such as acetone or methyl ethyl ketone, alkanolamines, pyridine, water, dimethyl formamide, or dimethyl sulfoxide.

Compounds of Formula (VI) Falling Within the Scope of the Invention.

A specific compound of formula VI is a compound wherein — is a single bond.

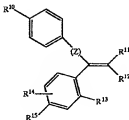
Another specific compound of formula VI is a compound wherein  $R^9$  and  $R_9$  together are  $-CH_2CH_2-$ ,  $-S-$ ,  $-O-$ ,  $-N(H)-$ ,  $-N(C_1-C_6\text{alkyl})-$ , or  $-CH=CH-$ .

Another specific compound of formula VI is a compound wherein — is  $-C(B)(D)-$ , wherein B and D are each halogen; and  $R^9$  and  $R_9$  are both hydrogen.

Another specific compound of formula VI is a compound wherein  $R^9$  is not phenyl or phenyl substituted by 1 or 2 V. Another specific compound of formula VI is a compound wherein  $R^9$  is not phenyl or phenyl substituted by 1 or 2 V. Another specific compound of formula VI is a compound wherein  $R^9$  is not phenyl, or phenyl substituted by 1 or 2 V.

A specific value for Z is  $-(CH_2)_{1-3}$ ,  $-O-$ ,  $-OCH_2-$ ,  $-CH_2O-$ ,  $-C(=O)O-$ ,  $-N(R_9)-$ , or a covalent bond. Another specific value for Z is  $-O-$ ,  $-OCH_2-$ ,  $-CH_2O-$ ,  $-C(=O)O-$ , or  $-N(R_9)-$ .

A specific compound of formula VI is a compound of formula VII:



wherein

Z is  $C=O$  or a covalent bond;

$R^{10}$  is mercapto,  $(C_1-C_6)$ alkylthio, hydroxy,  $(C_1-C_6)$ alkoxy,  $-O(CH_2)_{1-4}COOH$ ,  $-S(CH_2)_{1-4}COOH$ ,

$-(CH_2)_{1-4}COOH$ ,  $-O(CH_2)_{2-4}OH$ ,  $-S(CH_2)_{2-4}OH$ ,  $-O(CH_2)_{1-4}(C=O)R_9$ ,  $-S(CH_2)_{1-4}(C=O)R_9$ ,  $-O(CH_2)_{2-4}R_9$ ,  $-S(CH_2)_{2-4}R_9$ ,  $-(CH_2)_{1-4}R_9$ , or  $-(CH_2)_{1-4}(C=O)R_9$ ;

$R^{11}$  is 3-( $R_9$ )-4-( $R_9$ )phenyl, halo( $C_1-C_6$ )alkyl,  $(C_1-C_6)$ alkyl,  $(C_1-C_6)$ cycloalkyl,  $(C_1-C_6)$ alkylcyclo( $C_1-C_6$ )alkyl,  $(C_1-C_6)$ cycloalkenyl, or  $(C_1-C_6)$ alkyl( $C_1-C_6$ )cycloalkenyl;

$R^{12}$  is nitro, halo, ethyl, 2-cyanoethyl, 2-trifluoromethyl,  $-CH_2CH_2C(=O)O(C_1-C_6)$ alkyl, chloromethyl, cyclohexane, or naphthalene;

$R^{13}$  is H or together with  $R^{12}$  is  $O-CH=CH-$ ,  $-CH_2-CH_2-$  or  $-S-$ ;

$R^{14}$  is hydrogen, iodo,  $O(C_1-C_6)$ alkyl, hydroxy,  $-C(=O)O(C_1-C_6)$ alkyl,  $-OC(=O)(C_1-C_6)$ alkyl, benzyl, or  $OSO_2(CH_2)_{1-4}CH_3$ ;

$R^{15}$  is hydrogen,  $(C_1-C_6)$ alkyl, mercapto,  $(C_1-C_6)$ alkylthio, hydroxy,  $(C_1-C_6)$ alkoxy, iodo,  $OP(O)_2H_2$ ,  $-OSO_2(CH_2)_{1-4}CH_3$ ,  $-C(=O)O(C_1-C_6)$ alkyl,  $-OC(=O)(C_1-C_6)$ alkyl, or benzyl;

$R_9$  is amino, optionally substituted with one or two  $(C_1-C_6)$ alkyl; or  $R_9$  is an N-heterocyclic ring which optionally comprises another hetero atom selected from N, O, or S in said ring;

$R_9$  is hydrogen, halo, or hydroxy; and

$R_9$  is hydrogen,  $(C_1-C_6)$ alkyl, mercapto,  $(C_1-C_6)$ alkylthio, hydroxy,  $(C_1-C_6)$ alkoxy,  $-OSO_2-(CH_2)_{1-4}CH_3$ , halo,  $-OC(=O)(C_1-C_6)$ alkyl, or benzyl;

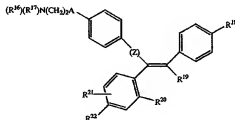
the compound is MER25, zindoxifene, DDAC (Analog II) or DTAC (102b);

a pharmaceutically acceptable salt thereof, or mixtures thereof.

A preferred compound of formula VII useful in the present invention is a compound wherein  $R^{14}$  is at the 5-position of the phenyl ring to which it is attached.

Another specific compound of formula (VI) useful in the present invention is a compound of formula (VIII):

(VIII)



wherein

A is O or S;

Z is  $C=O$  or a covalent bond;

$R^{16}$  and  $R^{17}$  are individually  $(C_1-C_6)$ alkyl or together with N are a saturated heterocyclic ring, preferably a 5-7 membered heterocyclic ring optionally containing 1-2 additional N( $R_{10}$ ), S or nonperoxide O, wherein  $R_{10}$  is hydrogen,  $(C_1-C_6)$ alkyl, phenyl or benzyl;

$R^{18}$  is hydrogen,  $(C_1-C_6)$ alkyl, mercapto,  $(C_1-C_6)$ alkylthio, hydroxy,  $(C_1-C_6)$ alkoxy;

$R^{19}$  is nitro, halo, ethyl, 2-cyanoethyl,

2-trifluoromethyl,  $-CH_2CH_2C(=O)O(C_1-C_6)$ alkyl,

or chloromethyl;

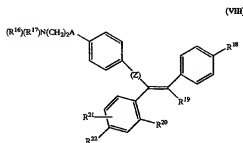
R<sup>20</sup> is H or together with R<sup>19</sup> is —CH<sub>2</sub>—CH<sub>2</sub>— or —S—;

R<sup>21</sup> is hydrogen, iodo, hydroxy, or O(C<sub>1</sub>–C<sub>4</sub>)alkyl; R<sup>22</sup> is hydrogen, (C<sub>1</sub>–C<sub>4</sub>)alkyl, mercapto, (C<sub>1</sub>–C<sub>4</sub>)alkylthio, hydroxy, (C<sub>1</sub>–C<sub>4</sub>)alkoxy, halo, or OPO<sub>3</sub>H<sub>2</sub>;

the compound is MER25, zindoxifene, DDAC (Analog II) or DTAC (102b);

a pharmaceutically acceptable salt thereof, or mixtures thereof.

Another specific compound of formula (VI) useful in the present invention is a compound of formula (VIII):



wherein

A is O;

Z is C=O or a covalent bond;

R<sup>16</sup> and R<sup>17</sup> are individually (C<sub>1</sub>–C<sub>4</sub>)alkyl or together with N are a saturated heterocyclic ring, preferably a 5–7 membered heterocyclic ring optionally containing 1–2 additional N(R), S or nonperoxide O, wherein R<sub>x</sub> is hydrogen, (C<sub>1</sub>–C<sub>4</sub>)alkyl, phenyl or benzyl;

R<sup>18</sup> is hydrogen, hydroxy, (C<sub>1</sub>–C<sub>4</sub>)alkyl, or (C<sub>1</sub>–C<sub>4</sub>)alkoxy;

R<sup>19</sup> is nitro, halo, ethyl or chloroethyl;

R<sup>20</sup> is H or together with R<sup>19</sup> is —CH<sub>2</sub>—CH<sub>2</sub>— or —S—;

R<sup>21</sup> is hydrogen, iodo, hydroxy, or (C<sub>1</sub>–C<sub>4</sub>)alkoxy;

R<sup>22</sup> is iodo, OPO<sub>3</sub>H<sub>2</sub>, (C<sub>1</sub>–C<sub>4</sub>)alkoxy or hydrogen; the compound is MER25, zindoxifene, DDAC (Analog II) or DTAC (102b);

a pharmaceutically acceptable salt thereof, or mixtures thereof.

A preferred compound of formula VIII useful in the present invention is a compound wherein Z is a covalent bond; R<sup>16</sup> and R<sup>17</sup> are each (C<sub>1</sub>–C<sub>4</sub>)alkyl or —(CH<sub>2</sub>)<sub>m</sub>—; R<sup>18</sup> is hydrogen; R<sup>21</sup> is hydrogen or iodo; and m is 4–6.

A preferred compound of formula VIII useful in the present invention is a compound wherein R<sup>19</sup> is ethyl or chloroethyl.

A preferred compound useful in the present invention is a compound of formula VIII wherein R<sup>19</sup> and R<sup>20</sup> together are —CH<sub>2</sub>—CH<sub>2</sub>—, and R<sup>22</sup> is OCH<sub>3</sub>.

A preferred compound of formula VIII useful in the present invention is a compound wherein:

Z is C=O or a covalent bond;

R<sup>16</sup> and R<sup>17</sup> are individually (C<sub>1</sub>–C<sub>4</sub>)alkyl or together with N are a saturated heterocyclic ring, preferably a 5–7 membered heterocyclic ring optionally comprising 1–2 additional N(R), S or nonperoxide O, wherein R<sub>x</sub> is hydrogen, (C<sub>1</sub>–C<sub>4</sub>)alkyl, phenyl or benzyl;

R<sup>18</sup> is hydrogen, hydroxy or O(C<sub>1</sub>–C<sub>4</sub>)alkyl;

R<sup>19</sup> is ethyl or chloroethyl;

R<sup>20</sup> is H or together with R<sup>19</sup> is —CH<sub>2</sub>—CH<sub>2</sub>— or —S—;

R<sup>21</sup> is hydrogen, iodo, hydroxy, or O(C<sub>1</sub>–C<sub>4</sub>)alkyl;

R<sup>22</sup> is iodo, OPO<sub>3</sub>H<sub>2</sub>, O(C<sub>1</sub>–C<sub>4</sub>)alkyl or hydrogen; a pharmaceutically acceptable salt thereof, or mixtures thereof.

Additionally for any compound of formula VIII or preferred compound of formula VIII described above, a specific value for R<sup>18</sup> is hydrogen; for Z is a covalent bond; for R<sup>16</sup> and R<sup>17</sup> is independently (C<sub>1</sub>–C<sub>4</sub>)alkyl, or for R<sup>16</sup> and R<sup>17</sup> taken together is —(CH<sub>2</sub>)<sub>m</sub>—; for R<sup>21</sup> is hydrogen or iodo; and for m is 4–6.

Additionally for any compound of formula VIII or preferred compound of formula VIII described above, a specific value for R<sup>22</sup> is OCH<sub>3</sub>; and for R<sup>19</sup> and R<sup>20</sup> together is —CH<sub>2</sub>—CH<sub>2</sub>—.

Compounds of formula VI useful in the present invention include tamoxifen and structural analogs of tamoxifen having substantial equivalent bioactivity. Such analogs include idoxifene, raloxifene, droloxifene, 3-iodotamoxifen, 4-iodotamoxifen, toremifene, trioxifene, nafoxifene, 4-hydroxytamoxifen, H-1285, and pharmaceutically acceptable salts thereof. A preferred embodiment of the invention is a compound of formula (VIII) wherein R<sup>19</sup> is not ethyl when R<sup>20</sup>, R<sup>21</sup>, and R<sup>22</sup> are H.

The term "structural analogs thereof" with respect to tamoxifen includes, but is not limited to, all of the compounds of formula (VI) which are capable of enhancing, increasing or elevating the level of TGF-beta. See, for example, U.S. Pat. Nos. 4,536,516, 5,457,113, 5,047,431, 5,441,986, 5,426,123, 5,384,332, 5,453,442, 5,492,922, 5,462,937, 5,492,926, 5,254,594 and U.K. Patent 1,064,629.

Because tamoxifen (TMX) causes liver carcinogenicity in rats and has been correlated with an increased risk of endometrial cancer in women and may increase the risk of certain gut cancers, other tamoxifen analogs may be considered safer to administer if they are less carcinogenic. The carcinogenicity of TMX has been attributed to the formation of covalent DNA adducts. Of the TMX analogs and derivatives, only TMX and toremifene have been studied for long-term carcinogenicity in rats. These studies provide strong evidence that covalent DNA adducts are involved in rodent hepatocarcinogenicity of TMX. Toremifene, which exhibits only a very low level of hepatic DNA adducts, was found to be non-carcinogenic. See Potter et al., *Carcinogenesis*, 15, 439 (1994).

It is postulated that 4-hydroxylation of TMX yields electrophilic alkylating agents which alkylate DNA through the ethyl group of TMX. This mechanistic hypothesis explains the low level of DNA adduct formation by the non-TMX analogs of formula (VI), including the TMX analog toremifene, and the absence of DNA adducts detected for the analogs 4-iodotamoxifen and idoxifene. Thus, all of these analogs are likely to be free from the risk of significant carcinogenesis in long term use. See Potter et al., supra. Idoxifene (IDX) includes (E)-1-[4-[2-(N-pyrrolidino)ethoxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene and its pharmaceutically acceptable salts and derivatives. See R. McCague et al., *Organic Preparations and Procedures Int.*, 26, 343 (1994) and S. K. Chandler et al., *Cancer Res.*, 51, 5851 (1991). Besides its lower potential for inducing carcinogenesis via formation of DNA adducts which can damage DNA, other advantages of IDX compared with TMX are that IDX has reduced residual estrogenic activity in rats and an improved metabolic profile.

Other "antisteroids" or "steroidal antagonists" are useful as TGF-beta activators or production stimulators or lead

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compounds, including other known stilbene-type antisteroids such as for example, *cis*- and *trans*-clomiphene, toremifene, cenchroman, raloxifene, droloxifene, 1-[4-(2-dimethylaminoethoxy)phenyl]-1-(3-hydroxyphenyl)-2-phenyl-2-butene (see U.S. Pat. No. 5,384,332), 1-nitro-1-phenyl-2-(4-hydroxyphenyl or anisyl)-2-[4-(2-pyrrol-N-ylthoxy)-phenyl]ethylene (CN-55,945), *trans*-1,2-dimethyl-1,2-(4-hydroxyphenyl)ethylene (*trans*-dimethylstilboestrol), *trans*-diethylstilboestrol, and 1-nitro-1-phenyl-2-(4-hydroxyphenyl)-2-[4-(3-dimethylaminopropoxy)phenyl]ethylene (GI680), metabolites or pharmaceutically acceptable salts thereof.

Known 1,2-diphenylethane-type antisteroids include *cis*-1,2-anisyl-1-[4-(2-dimethylaminoethoxy)phenyl]ethane (MRL-37), *trans*-1-[4-(4-chlorophenyl)-1,4-(2-dimethylaminoethoxy)phenyl]-2-phenylethanol (WSM-4613), 1-phenyl-1-[4-(2-dimethylaminoethoxy)phenyl]-2-anisylethanol (MER-25), 1-phenyl-1-[4-(2-dimethylaminoethoxy)phenyl]-2-anisylethane, mesobutoestrol (*trans*-1,2-dimethyl-1,2-(4-hydroxyphenyl)ethane), meso-hexestrol, (+)hexestrol and (-)hexestrol.

Known naphthalene-type antisteroids include nafoxidine, 1-[4-(2,3-dihydroxypropoxy)phenyl]-2-phenyl-6-hydroxy-1,2,3,4-tetrahydro-naphthalene, 1-[4-(4-hydroxyphenyl)-2-phenyl-6-hydroxy-1,2,3,4-tetrahydronaphthalene, 1-[4-(2-pyrrol-N-ylthoxy)-phenyl]-2-phenyl-6-methoxy-3,4-dihydronaphthalene (U11, 100A), and 1-[4-(2,3-dihydroxypropoxy)phenyl]-2-phenyl-6-methoxy-3,4-dihydronaphthalene (U-23, 469).

Known antisteroids which do not fall anywhere within these structural classifications include coumestrol, biochanin-A, genistein, methallenstril, phenocytin, and 1-[4-(2-dimethylaminoethoxy)phenyl]-2-phenyl-5-methoxyindene (U, 11555). In the nomenclature employed hereinabove, the term "anisyl" is intended to refer to a 4-methoxyphenyl group.

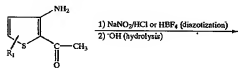
#### Preparation of a Compound of Formula (I-V)

Generally, a compound of formula I wherein  $R^4$  is  $(=O)_n$ , and  $n$  is 0 may be prepared by processes which are well known in the chemical arts for the synthesis of thiophene compounds and other aromatic compounds.

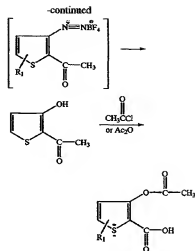
A compound of formula I wherein  $R^4$  is  $(=O)_n$ , and  $n$  is 1 or 2 can be prepared from a corresponding compound of formula II wherein  $n$  is 0, by oxidation of the thiophene sulfur using standard oxidation conditions.

Compounds of formula I wherein  $R^4$  is  $(C_1-C_6)$ alkyl,  $(C_1-C_6)$ alkenyl or  $(C_2-C_6)$ alkynyl and forms a sulfonium salt with the thiophene sulfur, wherein the associated counter ion is a pharmaceutically acceptable anion, can be prepared from corresponding compounds of formula I wherein  $R^4$  is  $(=O)_n$ , and  $n$  is 0 by alkylation or acylation of the thiophene sulfur, using procedures which are well known in the art.

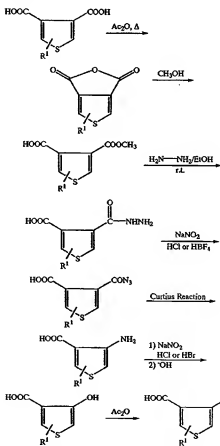
The synthesis of a compound of formula (II) can be carried out as follows:



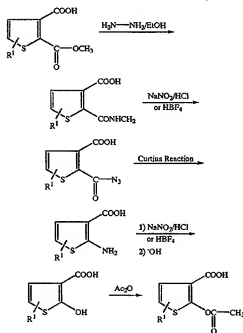
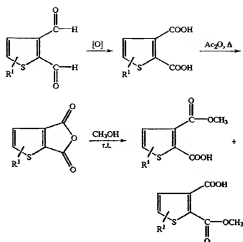
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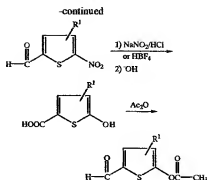
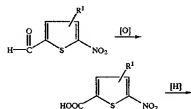
The synthesis of a compound of formula (III) may be carried out as follows:



The synthesis of a compound of formula (IV) may be carried out as follows:



The synthesis of a compound of formula (V) may be carried out as follows:



#### Preparation of Compounds of Formula (VI)

Generally, compounds of formula (VI) may be prepared using synthetic techniques which are analogous to techniques known in the art, including techniques described in R. A. Magarian, *Current Medicinal Chemistry*, 1994, 1, 61-104 and techniques described in the references relating to tamoxifen analogs which are cited and incorporated herein.

It may be convenient to optionally use a conventional protecting group during the preparation of compounds of formula (I) or compounds of formula (VI). The protecting group may be removed at an appropriate time during the synthesis, such as for example, when the final compound is to be formed. Such processes and intermediates for the manufacture of a compound of formula I are provided as further features of the invention.

#### Pharmaceutically Acceptable Acid and Base Addition Salts

The compounds used in the methods of the invention form pharmaceutically acceptable acid and base addition salts with a wide variety of organic and inorganic acids and bases and include the physiologically acceptable salts which are often used in pharmaceutical chemistry. Such salts are also part of this invention. Typical inorganic acids used to form such salts include hydrochloric, hydrobromic, hydroiodic, nitric, sulfuric, phosphoric, hypophosphoric and the like. Salts derived from organic acids, such as aliphatic mono and dicarboxylic acids, phenyl substituted alkanic acids, hydroxyalkanoic and hydroxyalanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids, may also be used. Such pharmaceutically acceptable salts thus include acetate, phenylacetate, trifluoroacetate, acrylate, ascorbate, benzoate, chlorobenzoate, dinitrobenzoate, hydroxybenzoate, methoxybenzoate, methylbenzoate, o-acetoxybenzoate, naphthalene-2-benzoate, bromide, isobutyrate, phenylbutyrate,  $\beta$ -hydroxybutyrate, butyne-1,4-dioate, hexyne-1,4-dioate, caprate, caprylate, chloride, cinnamate, citrate, formate, fumarate, glycolate, heptanoate, hippurate, lactate, malate, maleate, methoxymaleate, misonate, mandelate, mesylate, nicotinate, isonicotinate, nitrate, oxalate, phthalate, terphthalate, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate, propionate, propionate, phenylpropionate, salicylate, sebacate, succinate, suberate, sulfate, bisulfate, pyrosulfate, sulfite, bisulfite, sulfonate, benzene-sulfonate, p-bromophenylsulfonate, chlorobenzene-sulfonate, ethanesulfonate, 2-hydroxyethanesulfonate, methanesulfonate, naphthalene-1, sulfonate, naphthalene-2-sulfonate, p-toluenesulfonate, xylenesulfonate, tartrate, and the like. A preferred salt is the hydrochloride salt.

The pharmaceutically acceptable acid addition salts are typically formed by reacting a compound of formula (I) or

(VI) with an equimolar or excess amount of acid. The bases are generally combined in a mutual solvent such as diethyl ether or benzene. The salt normally precipitates out of solution within about one hour to 10 days and can be isolated by filtration or the solvent can be stripped off by conventional means.

Bases commonly used for formation of acid salts include ammonium hydroxide and alkali and alkaline earth metal hydroxide, carbonates, as well as aliphatic and primary, secondary and tertiary amines, aliphatic diamines. Bases especially useful in the preparation of addition salts include ammonium hydroxide, potassium carbonate, methylamine, diethylamine, ethylene diamine, cyclohexylamine and ethanolamine.

The pharmaceutically acceptable salts generally have enhanced solubility characteristics compared to the compound from which they are derived, and thus are often more amenable to formulation as liquids or emulsions, and can have enhanced bioavailability.

Identification of Therapeutic Agents Falling within the Scope of the Invention

Therapeutic agents useful in the practice of the invention, i.e., agents that elevate or increase TGF- $\beta$  levels, can be identified by an *in vitro* assay described in copending U.S. application Ser. No. 08/476,735, the disclosure of which is incorporated by reference herein, and/or the assays described in the Examples hereinbelow. It is recognized that not all therapeutic agents, e.g., aspirin, can increase TGF- $\beta$  levels. Moreover, it is recognized that some therapeutic agents within the scope of the invention increase TGF- $\beta$  levels to a greater extent than other TGF- $\beta$  elevating agents, however, methods to determine whether an agent falls within the scope of the invention are described hereinbelow.

The amounts of latent and/or active TGF- $\beta$  present in a sample of physiological fluid, such as a blood fraction, before and/or after the administration of the therapeutic agent, can be measured by methods disclosed in copending U.S. application Ser. No. 08/477,393 and U.S. Pat. No. 5,545,569, issued Aug. 13, 1995, the disclosures of which are incorporated by reference herein.

For example, to determine whether an agent can elevate levels of TGF- $\beta$ , an agent or mixture of agents is first tested on rat aortic vascular smooth muscle cells (rVSMCs) for their ability to stimulate the production of active TGF- $\beta$  in the culture medium as originally described for tamoxifen. See Grainger et al. (*Biochem. J.*, 294, 109 (1993)). The key step in demonstrating that cells have a reduced proliferation rate as a result of TGF- $\beta$  production and activation is that the effect can be fully reversed by neutralizing antibodies to TGF- $\beta$ . Incomplete reversal of a decreased rate of proliferation is evidence for TGF- $\beta$  independent effect(s), which may include toxicity.

The effects of an agent are then tested on explant human aortic smooth muscle cells (hVSMC) to determine whether the agent also stimulates production of TGF- $\beta$  by these cells. The use of explant hVSMCs, is essential because (i) explant hVSMCs grown under non-optimal conditions (particularly at low cell densities) will spontaneously produce TGF- $\beta$ ; (ii) bVSMC cultures from cells prepared by enzyme dispersal spontaneously produce substantial amounts of TGF- $\beta$  in culture (Kirschner et al., *Am. J. Physiol.*, 265, C571 (1993)) and therefore cannot be used for screening; and (iii) the sensitivity of rVSMCs and bVSMCs to agents which induce the cells to produce TGF- $\beta$  differs by up to 100-fold.

In screening for agents likely to be effective for clinical purposes, it is therefore necessary to use bVSMCs to deter-

mine both potency and the therapeutic window between effective concentrations and toxic concentrations for human cells. Candidate agents which pass the *in vitro* cell culture screens are then tested on one or more animal models of vascular conditions or disease, e.g., animal models of atherosclerosis include lipid lesion formation in C57B16 mice and mice expressing the human apo(A) transgene that are fed a high fat diet, apoE knockout mice fed a normal diet, or cholesterol-fed Watanabe rabbits.

To determine total TGF- $\beta$ , ELISA plates are coated with a chicken antibody that binds both latent and active TGF- $\beta$ . Patient sera or plasma are incubated with these ELISA plates, then the plates are washed to remove unbound components of the patients' sera or plasma. Rabbit anti-TGF- $\beta$  antibody, capable of binding both latent and active TGF- $\beta$ , is then added to the plates and incubated. The plates are then washed to remove unbound antibody, and peroxidase-labeled anti-rabbit IgG is added. After incubation and washing, the plates are exposed to the chromogenic substrate, ortho-phenylenediamine. The presence of total TGF- $\beta$  in patients' sera or plasma is then determined calorimetrically at  $A_{492}$  by comparison to a standard curve. In patients treated with an agent that modifies TGF- $\beta$ , a pretreatment determination of TGF- $\beta$  can be compared with post-treatment time points to monitor treatment results and effectiveness.

In an alternate format, TGF- $\beta$  type II receptor extracellular domain, which recognizes the active form(s) of TGF- $\beta$ , but not the mature or latent forms, is coated onto ELISA plates. Patient sera or plasma are added to the plates, and processed as above. This assay measures active TGF- $\beta$  present in sera or plasma.

In another alternate format, fluorescent-labeled anti-TGF- $\beta$  antibody is used in place of peroxidase labeled second antibody to detect the presence of TGF- $\beta$  in patients' sera or plasma. In yet another alternate format, anti-TGF- $\beta$  antibody is labeled with a radioactive moiety capable of detection by standard means. These latter two assays may be performed in an ELISA format, with or without using the additional anti-TGF- $\beta$  antibody described above.

It is envisioned that the therapeutic agents of the invention can increase TGF- $\beta$  levels by increasing the number of TGF- $\beta$  transcripts, increasing the translational efficiency of TGF- $\beta$  transcripts, increasing the post-translational processing of the latent form of TGF- $\beta$  to the active form of TGF- $\beta$ , increasing the bioavailability of TGF- $\beta$ , and/or increasing the biological effect of active TGF- $\beta$ , e.g., by increasing the affinity of TGF- $\beta$  for its receptor, increasing the affinity of the receptor for TGF- $\beta$  and/or by increasing the number of receptors for TGF- $\beta$  on the cell surface, or any combination thereof. For example, the administration of aspirin or copper aspirinate (see Examples III and IV) can increase the level of latent TGF- $\beta$  in a mammal relative to the level of latent TGF- $\beta$  in that mammal prior to aspirin or copper aspirinate administration.

Agents useful in the practice of the methods of the invention can also be identified by the correlation of agent administration with the inhibition or reduction in atherosclerotic plaque development or formation, an increase in lesion regression or plaque stability, or a decrease vascular wall hypertrophy and/or hyperplasia *in vivo*. Agent efficacy is measured by methods available to those skilled in the art including, but not limited to, angiography, ultrasonic evaluation, fluorescent imaging, fiber optic endoscopic examination or biopsy and histology. The activity of the therapeutic agents of the invention *in vivo* can also be monitored indirectly by the measurement of the levels of

TGF-beta in a patient before and after the administration of the therapeutic agent.

For non-vascular indications, agents useful in the practice of the invention can be identified by the correlation of in vivo agent administration with a reduction in a particular pathology associated with the non-vascular indication. For example, animal models for multiple sclerosis (Martin et al., *Ann. Rev. Immunol.*, 10, 153 (1992); Hafler et al., *Immunol. Today*, 10, 107 (1989); WO 93/16724) and rheumatoid arthritis (WO 93/16724) may be employed to determine the activity of the therapeutic agents of the invention in vivo. Additionally, suitable animal models for osteoporosis (suspension induced osteoporosis in rats) and cancer (DMBA-induced skin cancer) are well known in the art. Vascular indications useful to Treatment by the claimed Methods

The therapeutic agents of the invention are useful to treat a mammal such as a human patient, afflicted with, or at risk of, a vascular indication. In particular, the therapeutic agents of the invention are useful to treat a mammal afflicted with, or at risk of, a vascular indication associated with a deficiency in TGF-beta.

A mammal afflicted with, or at risk of, a vascular indication that would benefit from the practice of the claimed invention includes a mammal exhibiting a reduced level of TGF-beta within the vessel wall. Such mammals may be identified as having one or more risk factors which contribute to reduced TGF-beta activity. These factors include low serum active levels of TGF-beta, elevated circulating PAI-1 antigen or activity, elevated circulating lipoprotein (a), elevated blood concentration of LDL and/or VLDL in the fasting state, the ability to elevate PAI-1 following a fat tolerance test, the presence of the 4G allele of the PAI-1 promoter, and the like. Thus, the measurement of PAI-1/TGF-beta response (Example 7) to fat feeding is one method to determine whether an individual is at risk of a vascular indication associated with a deficiency in TGF-beta levels. For example, low serum active TGF-beta levels can be levels that are less than about 4 ng/ml, preferably less than about 3 ng/ml, and more preferably less than about 2 ng/ml. Dosages, Formulations and Routes of Administration of the Therapeutic Agents of the Invention

Aspirin or aspirinates, e.g., the compounds of formula (I), and the aspirinate salts of the invention, including their coordination solvates, are preferably administered at doses of about 0.001-600 mg/kg, more preferably at doses of about 2.0-165 mg/kg, and even more preferably at doses of about 1.0-100 mg/kg of body weight, although other dosages may provide beneficial results.

Fish oil, a source of omega-3 fatty acids, is administered at doses of about 200-18000 mg/kg/day, more preferably at doses of about 1000-6000 mg/kg/day, and even more preferably at doses of about 1200-4000 mg/kg/day, although other dosages may provide beneficial.

For compounds of the formula (VI), generally, accepted and effective daily doses will be from about 0.05 mg/kg/day to about 10 mg/kg/day, preferably about 0.1-1.0 mg/kg/day, more preferably about 0.3-0.5 mg/kg/day. For local delivery, an exemplary dose will be about 0.01 to about 1000 µg/ml, preferably followed by a chronic lower dose, which is preferably administered orally. It is also contemplated that a large loading dose may be employed, e.g., about 1.0 to about 100 mg/kg, to rapidly establish a therapeutic level of the agent. The large loading dose is preferably followed by a chronic dose of about 0.1 to about 20 mg/kg/day, preferably about 0.5 to about 2 mg/kg/day. It is preferred that a compound of formula (VI) is administered in the form of an

acid addition salt, as is customary in the administration of pharmaceuticals comprising a basic group, such as an amino or N-heterocyclic group.

The amount of therapeutic agent administered is selected to treat a particular vascular indication. For example, to treat vascular traumas of differing severity, smaller doses are sufficient to treat lesser vascular trauma, such as to prevent vascular rejection following graft or transplant, while larger doses are sufficient to treat more extensive vascular trauma, such as restenosis following angioplasty. The therapeutic agents of the invention are also amenable to chronic use for prophylactic purposes to treat disease states involving proliferation of vascular smooth muscle cells and pericytes derived from the medial layers of vessels, pericytes and fibroblasts in the adventitia, and migrating macrophage/mesenchymal foam cells, over time (e.g., atherosclerosis, coronary heart disease, thrombosis, myocardial infarction, stroke, uterine fibroid or fibroses and the like), preferably by systemic administration.

Administration of the therapeutic agents in accordance with the present invention may be continuous or intermittent, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is therapeutic or prophylactic, and other factors known to skilled practitioners. The administration of the agents of the invention may be essentially continuous over a preselected period of time or may be in a series of spaced doses, e.g., either before, during, or after procedural vascular trauma, before and during, before and after, during and after, or before, during and after the procedural trauma.

One or more suitable unit dosage forms comprising the therapeutic agents of the invention, which, as discussed below, may be formulated for sustained release, can be administered by a variety of routes including oral, or parenteral, including by rectal, transdermal, subcutaneous, intravenous, intramuscular, intrapulmonary and intranasal routes. When the therapeutic agents of the invention are prepared for oral administration, they are preferably combined with a pharmaceutically acceptable carrier, diluent or excipient to form a pharmaceutical formulation, or unit dosage form. The total active ingredients in such formulations comprise from 0.1 to 99.9% by weight of the formulation. By "pharmaceutically acceptable" it is meant the carrier, diluent, excipient, and/or salt must be compatible with the other ingredients of the formulation, and not deleterious to the recipient thereof.

Pharmaceutical formulations containing the therapeutic agents of the invention can be prepared by procedures known in the art using well known and readily available ingredients. For example, a copper aspirinate including copper 2-acetylsalicylate, or a compound of formula (I), as well as a compound of formula (VI), can be formulated with common excipients, diluents, or carriers, and formed into tablets, capsules, suspensions, powders, and the like. Examples of excipients, diluents, and carriers that are suitable for such formulations include the following fillers and extenders such as starch, sugars, mannitol, and silicic derivatives; binding agents such as carboxymethyl cellulose, HPMC, and other cellulose derivatives, alginates, gelatin, and polyvinyl-pyrrolidone; moisturizing agents such as glycerol; disintegrating agents such as calcium carbonate and sodium bicarbonate; agents for retarding dissolution such as paraffin; resorption accelerators such as quaternary ammonium compounds; surface active agents such as cetyl alcohol, glycerol monoacetate; adsorptive carriers such as kaolin and bentonite; and lubricants such as talc, calcium and magnesium stearate, and solid polyethyl glycols.

For example, tablets or caplets containing aspirinates of the invention can include buffering agents such as calcium carbonate, magnesium oxide and magnesium carbonate. Caplets and tablets can also include inactive ingredients such as cellulose, pregelatinized starch, silicon dioxide, hydroxypropyl methylcellulose, magnesium stearate, microcrystalline cellulose, starch, talc, titanium dioxide, benzoic acid, citric acid, corn starch, mineral oil, polypropylene glycol, sodium phosphate, and zinc stearate, and the like. Hard or soft gelatin capsules containing aspirinates of the invention can contain ingredients such as gelatin, microcrystalline cellulose, sodium lauryl sulfate, starch, talc, and titanium dioxide, and the like, as well as liquid vehicles such as polyethylene glycols (PEGs) and vegetable oil. Moreover, the enteric coated caplets or tablets of the copper aspirinates of the invention are designed to resist disintegration in the stomach and dissolve in the more neutral to alkaline environment of the duodenum.

The pharmaceutical formulations of the therapeutic agents of the invention can take the form of an aqueous or anhydrous solution or dispersion, or alternatively the form of an emulsion or suspension. The therapeutic agents of the invention can also be formulated as elixirs or solutions for convenient oral administration or as solutions appropriate for parenteral administration, for instance by intramuscular, subcutaneous or intravenous routes.

These formulations can contain pharmaceutically acceptable vehicles and adjuvants which are well known in the prior art. It is possible, for example, to prepare solutions using one or more organic solvents which is/are acceptable from the physiological standpoint, chosen, in addition to water, from solvents such as acetone, ethanol, isopropyl alcohol, glycol ethers such as the products sold under the name "Dowanol", polyglycols and polyethylene glycols, C<sub>1</sub>-C<sub>4</sub> alkyl esters of short-chain acids, preferably ethyl or isopropyl lactate, fatty acid triglycerides such as the products marketed under the name "Miglyol", isopropyl myristate, animal, mineral and vegetable oils and polysiloxanes.

The compositions according to the invention can also contain thickening agents such as cellulose and/or cellulose derivatives. They can also contain gums such as xanthan, alginates, guar, or carbo gum or gum arabic, or alternatively thickeners such as polyethylene glycols, bentones and montmorillonites, and the like.

It is possible to add, if necessary, an adjuvant chosen from antioxidants, surfactants, preservatives, film-forming, keratolytic or comedolytic agents, perfumes and colorings. Also, other active ingredients may be added, whether for the conditions described or some other condition.

For example, among antioxidants, *t*-butylhydroquinone, butylated hydroxyanisole, butylated hydroxytoluene and  $\alpha$ -tocopherol and its derivatives may be mentioned. The galenic forms chiefly conditioned for topical application take the form of creams, milks, gels, dispersion or microemulsions, lotions thickened to a greater or lesser extent, impregnated pads, ointments or sticks, or alternatively the form of aerosol formulations in spray or foam form or alternatively in the form of a cake of soap.

Additionally, the agents are well suited to formulation as sustained release dosage forms and the like. The formulations can be so constituted that they release the active ingredient only or preferably in a particular part of the intestinal tract, possibly over a period of time. The coatings, envelopes, and protective matrices may be made, for example, from polymeric substances or waxes.

The therapeutic agents of the invention can be delivered via patches for transdermal administration. See U.S. Pat. No.

5,560,922 for examples of patches suitable for transdermal delivery of a therapeutic agent. Patches for transdermal delivery can comprise a backing layer and a polymer matrix which has dispersed or dissolved therein a therapeutic agent effective for reducing vessel lumen diameter diminution, along with one or more skin permeation enhancers. The backing layer can be made of any suitable material which is impermeable to the therapeutic agent. The backing layer serves as a protective cover for the matrix layer and provides also a support function. The backing can be formed so that it is essentially the same size layer as the polymer matrix or it can be of larger dimension so that it can extend beyond the side of the polymer matrix or overlay the side or sides of the polymer matrix and then can extend outwardly in a manner that the surface of the extension of the backing layer can be the base for an adhesive means. Alternatively, the polymer matrix can contain, or be formulated of, an adhesive polymer, such as polyacrylate or acrylate/vinyl acetate copolymer. For long-term applications it might be desirable to use microporous and/or breathable backing laminates, so hydration or maceration of the skin can be minimized.

Examples of materials suitable for making the backing layer are films of high and low density polyethylene, polypropylene, polyurethane, polyvinylchloride, polyesters such as poly(ethylene phthalate), metal foils, metal foil laminates of such suitable polymer films, and the like. Preferably, the materials used for the backing layer are laminates of such polymer films with a metal foil such as aluminum foil. In such laminates, a polymer film of the laminate will usually be in contact with the adhesive polymer matrix.

The backing layer can be any appropriate thickness which will provide the desired protective and support functions. A suitable thickness will be from about 10 to about 200 microns.

Generally, those polymers used to form the biologically acceptable adhesive polymer layer are those capable of forming shaped bodies, thin walls or coatings through which therapeutic agents can pass at a controlled rate. Suitable polymers are biologically and pharmaceutically compatible, nonallergenic and insoluble in and compatible with body fluids or tissues with which the device is contacted. The use of soluble polymers is to be avoided since dissolution or erosion of the matrix by skin moisture would affect the release rate of the therapeutic agents as well as the capability of the dosage unit to remain in place for convenience of removal.

Exemplary materials for fabricating the adhesive polymer layer include polyethylene, polypropylene, polyurethane, ethylene/propylene copolymers, ethylene/ethylacrylate copolymers, ethylene/vinyl acetate copolymers, silicone elastomers, especially the medical-grade polydimethylsiloxanes, neoprene rubber, polyisobutylene, polyacrylates, chlorinated polyethylene, polyvinyl chloride, vinyl chloride-vinyl acetate copolymers, crosslinked polymethacrylate polymers (hydrogel), polyvinylidene chloride, poly(ethylene terephthalate), butyl rubber, epichlorohydrin rubbers, ethylenevinyl alcohol copolymers, ethylene-vinylalcohol copolymers; silicone copolymers, for example, polysiloxane-polycarbonate copolymers, polysiloxane-polyethylene oxide copolymers, polysiloxane-polydimethylsiloxane copolymers, polysiloxane-alkylene copolymers (e.g., polysiloxane-ethylene copolymers), polysiloxane-alkylenesilane copolymers (e.g., polysiloxane-ethylenesilane copolymers), and the like; cellulose polymers, for example methyl or ethyl cellulose, hydroxypropyl methyl cellulose, and cellulose esters; polycarbonates; polytetrafluoroethylene; and the like.

Preferably, a biologically acceptable adhesive polymer matrix should be selected from polymers with glass transition temperatures below room temperature. The polymer may, but need not necessarily, have a degree of crystallinity at room temperature. Cross-linking monomeric units or sites can be incorporated into such polymers. For example, cross-linking monomers can be incorporated into polyacrylate polymers, which provide sites for cross-linking the matrix after dispersing the therapeutic agent into the polymer. Known cross-linking monomers for polyacrylate polymers include polymethacrylic esters of polyols such as butylene diacrylate and dimethacrylate, trimethylol propane trimethacrylate and the like. Other monomers which provide such sites include allyl acrylate, allyl methacrylate, diallyl maleate and the like.

Preferably, a plasticizer and/or humectant is dispersed within the adhesive polymer matrix. Water-soluble polyols are generally suitable for this purpose. Incorporation of a humectant in the formulation allows the dosage unit to absorb moisture on the surface of skin which in turn helps to reduce skin irritation and to prevent the adhesive polymer layer of the delivery system from failing.

Therapeutic agents released from a transdermal delivery system must be capable of penetrating each layer of skin. In order to increase the rate of permeation of a therapeutic agent, a transdermal drug delivery system must be able in particular to increase the permeability of the outermost layer of skin, the stratum corneum, which provides the most resistance to the penetration of molecules. The fabrication of patches for transdermal delivery of therapeutic agents is well known to the art.

The local delivery of the therapeutic agents of the invention can also be by a variety of techniques which administer the agent at or near the diseased or traumatized vascular site. Examples of site-specific or targeted local delivery techniques are not intended to be limiting but to be illustrative of the techniques available. Examples include local delivery catheters, such as an infusion or indwelling catheter, a needle catheter, elutriated and stents or other implantable devices, site specific carriers, direct injection, or direct applications. In addition, local delivery of the therapeutic agents to branch points may be particularly beneficial as active TGF beta levels are lower at branch points, where lesion formation is increased relative to non-branch points.

Catheters which may be useful in the practice of the invention include catheters such as those disclosed in Just et al. (U.S. Pat. No. 5,232,444), Abusio et al. (U.S. Pat. No. 5,213,576), Shapland et al. (U.S. Pat. No. 5,282,785), Racchini et al. (U.S. Pat. No. 5,458,568) and Shaffer et al. (U.S. Pat. No. 5,049,132), the disclosures of which are incorporated by reference herein.

For a compound of formula (VI), which may be administered in accordance with the present invention using an infusion catheter, such as produced by C.R. Bard Inc., Billerica, Mass., or that disclosed by Wolinsky (U.S. Pat. No. 4,824,436) or Spears (U.S. Pat. No. 4,512,762), a therapeutically/prophylactically effective dosage of the compounds of formula (VI) will be typically reached when the concentration thereof in the fluid space between the balloons of the catheter is in the range of about  $10^{-3}$  to  $10^{-12}$  M. The compounds of formula (VI) may only need to be delivered in an anti-proliferative therapeutic/prophylactic dosage sufficient to expose the proximal (6 to 9) cell layers of the intimal or tunica media cells lining the lumen thereto. Also, such a dosage can be determined empirically, e.g., by a) infusing vessels from suitable animal model systems and using immunohistochemical methods to detect the com-

pound of formula (VI) and its effects; and b) conducting suitable in vitro studies.

Local delivery by an implant involves the surgical placement of a matrix that contains the therapeutic agent at the lesion site or traumatized area. The implanted matrix releases the therapeutic agent by diffusion, chemical reaction, or solvent activators. Lange, *Science*, 249, 1527 (1990).

An example of targeted local delivery by an implant is the use of a stent. Stents are designed to mechanically prevent the collapse and reocclusion of the coronary arteries. Incorporating a therapeutic agent into the stent delivers the therapeutic agent directly to the lesion. Local delivery of agents by this technique is described in Koh, *Pharmaceutical Technology* (October, 1990).

For example, a metallic, plastic or biodegradable intravascular stent can be employed which comprises an effective amount of a therapeutic agent. The stent preferably comprises a biodegradable coating or a porous or permeable non-biodegradable coating comprising the therapeutic agent. A more preferred embodiment of the invention is a coated stent wherein the coating comprises a sustained-release dosage form of the therapeutic agent. In an alternative embodiment, a biodegradable stent may also have a therapeutic agent impregnated therein, i.e., in the stent matrix.

A biodegradable stent with the therapeutic agent impregnated therein can further be coated with a biodegradable coating or with a porous non-biodegradable coating having the sustained release-dosage form of the therapeutic agent dispersed therein. Such a stent can provide a differential release rate of the therapeutic agent, i.e., there can be a faster initial release of the therapeutic agent from the coating followed by a slower delayed release of the therapeutic agent impregnated in the stent matrix, upon degradation of the stent matrix. The intravascular stent also provides a mechanical means of providing an increase in luminal area of a vessel.

Furthermore, the placement of intravascular stents comprising a therapeutic agent which is an inhibitor of smooth muscle cell proliferation can provide increased efficacy by reducing or preventing intimal proliferation. This inhibition of intimal smooth muscle cells and stroma produced by the smooth muscle and pericytes can allow more rapid and complete re-endothelialization following the intravascular placement of the vascular stent. The increased rate of re-endothelialization and stabilization of the vessel wall following stent placement can reduce the loss of luminal area and decreased blood flow which is the primary cause of vascular stent failures.

Another example is a delivery system in which a polymer that contains the therapeutic agent is injected into the lesion in liquid form. The polymer then solidifies or cures to form the implant which is retained in situ. This technique is described in published PCT application WO 90/03768 (Dom, Apr. 19, 1990).

Another example is the delivery of a therapeutic agent by polymeric endoluminal sealing. This technique uses a catheter to apply a polymeric implant to the interior surface of the lumen. The therapeutic agent incorporated into the biodegradable polymer implant is thereby released at the surgical site. This technique is described in published PCT application WO 90/01969 (Schindler, Aug. 23, 1989), the disclosure of which is incorporated by reference herein.

Yet another example of local delivery by an implant is by direct injection of vesicles or microparticles into the lesion. These microparticles may be composed of sub-

stances such as proteins, lipids, carbohydrates or synthetic polymers. These microparticulates have the therapeutic agent incorporated throughout the microparticle or over the microparticle as a coating. Delivery systems incorporating microparticulates are described in Lange, *Science*, 249,1527 (1990) and Mathiowitz et al., *J. App. Poly Sci.*, 26, 809 (1981).

Local delivery by site specific carriers involves attaching the therapeutic agent to a carrier which will direct the therapeutic agent to the target site, i.e., to a proliferative lesion. Examples of this delivery technique includes the use of carriers such as a protein ligand, e.g., a monoclonal antibody or antibody fragment. Lange, *Science*, 249,1527 (1990).

Local delivery by direct application also includes applying the therapeutic agent directly to tissue, such as to the arterial bypass graft during the surgical procedure, or an artificial graft, and then implanting the treated graft or other tissue.

For topical administration, the therapeutic agents may be formulated as is known in the art for direct application to a target area. Conventional forms for this purpose include wound dressings, coated bandages or other polymer coverings, ointments, lotions, pastes, jellies, sprays, and aerosols. The percent by weight of a therapeutic agent of the invention present in a topical formulation will depend on various factors, but generally will be from 0.5% to 95% of the total weight of the formulation, and typically 1-25% by weight.

It will be recognized by those skilled in the art that therapeutically-prophylactically effective dosages of these therapeutic agents and compositions will be dependent on several factors. For example, with respect to catheter delivery, those factors include a) the atmospheric pressure applied during infusion; b) the time over which the agent administered resides at the vascular site; c) the form of the therapeutic or prophylactic agent employed; and/or d) the nature of the vascular trauma and therapy desired. Those skilled practitioners trained to deliver drugs at therapeutically or prophylactically effective dosages (e.g., by monitoring drug levels and observing clinical effects in patients) will determine the optimal dosage for an individual patient based on experience and professional judgment. Those skilled in the art will recognize that infiltration of the therapeutic agent into initial layers of a diseased or traumatized human vessel wall in free or sustained-release form is subject to variation and will need to be determined on an individual basis.

A further aspect of the invention provides a method of treating a mammal having, or at risk of, an indication associated with a TGF-beta deficiency comprising administering an amount of an aspirinate effective to elevate the level of TGF-beta.

A further aspect of the invention provides a method of treating a mammal having, or at risk of, a vascular indication which is associated with a TGF-beta deficiency, comprising administering an amount of an aspirinate effective to elevate the level of TGF-beta so as to inhibit or reduce the diminution of vessel lumen diameter. Specifically, the administration is effective to reduce or prevent lipid accumulation by the vessel, to increase plaque stability of an atherosclerotic lesion, to inhibit atherosclerotic lesion formation or development, or to induce atherosclerotic lesion regression.

A further aspect of the invention provides a method of treating a mammal having, or at risk of, an indication associated with a TGF-beta deficiency other than hypertension, thrombosis or atherosclerosis, comprising

administering an amount of 3-acetoxy-2-carboxythiophene or a pharmaceutically acceptable salt thereof, effective to elevate the level of TGF-beta.

A further aspect of the invention provides a therapeutic method for preventing or treating a condition or symptom associated with Parkinson's disease, Marfan's syndrome, Alzheimer's disease, senile dementia, osteoporosis, multiple sclerosis, lupus erythematosus, or fibrosis, comprising administering to a mammal in need of such therapy, an effective amount of an aspirinate.

A further aspect of the invention provides a therapeutic method for preventing or treating a condition or symptom associated with an auto-immune disease, comprising administering to a mammal in need of such therapy, an effective amount of an aspirinate, provided the aspirinate is not a copper salt of an aryl or heteroaryl carboxylic acid.

A further aspect of the invention provides a therapeutic method for lowering serum cholesterol, comprising administering to a mammal in need of such therapy, an effective amount of an aspirinate.

A further aspect of the invention provides a therapeutic method for enhancing or promoting wound healing, comprising administering to a mammal in need of such therapy, an effective amount of an aspirinate.

A further aspect of the invention provides a kit comprising, separately packaged, a device adapted for the local delivery of an agent to a site in the lumen of a vessel of a mammal, and at least one unit dosage form of an aspirinate, wherein the aspirinate is effective to maintain or elevate the TGF-beta levels in said mammal.

A further aspect of the invention provides a method of preventing or treating a mammal having, or at risk of developing, atherosclerosis, comprising administering an amount of a combination of aspirin or an aspirinate and at least one omega-3 fatty acid, wherein said amount is effective to maintain or increase the level of TGF-beta so as to inhibit or reduce vessel lumen diameter diminution.

A further aspect of the invention provides a pharmaceutical composition comprising: (a) an amount of a first agent effective to elevate the level of latent TGF-beta; and (b) an amount of a second agent effective to increase the level of TGF-beta which is capable of binding to the TGF-beta receptors.

A further aspect of the invention provides a pharmaceutical composition comprising: (a) an aspirinate; and (b) at least one omega-3 fatty acid; wherein components (a) and (b) are present in a combined amount effective to maintain or elevate TGF-beta levels when the composition is administered to a mammal.

A further aspect of the invention provides a kit comprising, separately packaged, a unit dosage form of a first agent effective to elevate the level of latent TGF-beta in a mammal, a unit dosage form of a second agent effective to increase the level of TGF-beta which is capable of binding to the TGF-beta receptors in said mammal, and a device adapted for the local delivery of at least one of said agents.

A further aspect of the invention provides a kit comprising, separately packaged, a device adapted for the local delivery of at least one agent to a site in the lumen of a mammalian vessel and at least one unit dosage of aspirin or an aspirinate and at least one unit dosage of at least one omega-3 fatty acid, and wherein either or both unit dosages are effective to maintain or elevate TGF-beta levels.

A further aspect of the invention provides a therapeutic method comprising: (a) identifying a patient exhibiting a decreased level of active TGF-beta and afflicted with a pathology associated with said decreased level; and (b)

administering to the patient an agent that elevates the level of TGF-beta which is capable of binding to the TGF-beta receptors so as to alleviate at least one of the symptoms of said pathology.

A further aspect of the invention provides a method comprising determining endothelial cell activation in a mammal by detecting immunoglobulins that specifically bind to a TGF- $\beta$  Type II receptor or a portion thereof.

A further aspect of the invention provides a method comprising diagnosing or monitoring a disease characterized by endothelial cell activation in a mammal by detecting immunoglobulins that specifically bind to a TGF- $\beta$  Type II receptor or a portion thereof.

A further aspect of the invention provides a method comprising diagnosing or monitoring atherosclerosis in a mammal by detecting immunoglobulins that bind to a TGF- $\beta$  Type II receptor or a portion thereof.

Specifically, in the above methods, immunoglobulins can be detected by: (a) combining a physiological fluid from the mammal which comprises the immunoglobulins, with a capture moiety that binds the immunoglobulins, forming a capture complex comprising the capture moiety and the immunoglobulins; and (b) detecting or determining the amount of the capture complex. The capture moiety can be immobilized. The capture moiety can comprise a TGF- $\beta$  Type II receptor or a portion thereof. The capture moiety can comprise the extracellular domain of the TGF- $\beta$  Type II receptor. The immunoglobulins can comprise anti-TGF- $\beta$  Type II receptor-IgG antibodies. The immunoglobulins can comprise anti-TGF- $\beta$  Type II receptor-IgD antibodies. The signal moiety can comprise anti-human-pan-IgG antibodies. The signal moiety can comprise anti-human-IgG subclass specific antibodies. The signal moiety can comprise anti-human-IgD antibodies. The physiological fluid can be serum, or plasma. The mammal can be a human. The immunoglobulins can bind to the extracellular domain of the TGF- $\beta$  Type II receptor. The immunoglobulins can comprise IgG antibodies. And, a signal moiety comprising a detectable label can detect the complex.

A further aspect of the invention provides a method comprising detecting mammalian cells having TGF- $\beta$  Type II receptors, by combining the cells with a capture moiety that binds TGF- $\beta$  Type II receptors or a portion thereof, forming a capture complex; and detecting or determining the amount of the capture complex. The method may optionally further comprising comparing the amount with a standard curve referenced to a pool of normal cells of tissue. The capture moiety can comprise immunoglobulins. The immunoglobulins can bind to the extracellular domain of the TGF- $\beta$  Type II receptor. The immunoglobulins can comprise IgG antibodies. The immunoglobulins can comprise IgD antibodies. Specifically, a signal moiety comprising a detectable label can detect the capture complex; the signal moiety can comprise anti-human-pan-IgG antibodies, anti-human-IgG subclass specific antibodies, or anti-human-IgD antibodies; and the cells can be brachial cells or femoral cells.

A further aspect of the invention provides a method comprising diagnosing or monitoring atherosclerosis in a mammal by detecting TGF- $\beta$  type II receptors in cells or tissue of the mammal.

A further aspect of the invention provides a kit comprising packaging material containing: a) a capture moiety comprising the extracellular domain of the TGF- $\beta$  Type II receptor; and b) a detection moiety capable of binding to an immunoglobulin. Specifically, the immunoglobulin can be IgG, or IgD.

The invention will be better understood by making reference to the following specific examples.

## EXAMPLE I

## Association of TGF-beta with Lipoprotein Particles

TGF-beta is a hydrophobic protein known to have affinity for polymeric aliphatic hydrocarbons. To determine whether TGF-beta would associate with lipoprotein particles in the circulation, platelet-poor plasma was prepared from peripheral venous blood drawn from ten healthy donors (A-I) and two donors with diabetes (K and L). The absence of platelet degradation (<0.02% degradation) was confirmed by measurement of PF-4 (platelet factor-4) in the plasma by ELISA (Asserchom PF-4; Diagnostic Stago, FR). A 1 ml aliquot of plasma was diluted to 4 ml with Buffer A (Havel et al., *J. Clin. Invest.*, 34, 1345 (1955)) and then KBr was added to final density of 1.215 g/ml. The lipoproteins were separated from the plasma proteins by density gradient ultracentrifugation (235,000g) at 4° C. for 48 hours. The top 2 ml was collected as the lipoprotein fraction and the lower 2 ml was collected as the lipoprotein deficient plasma fraction.

The total cholesterol in each fraction was measured by the cholesterol oxidase enzymatic method (Sigma Diagnostics) as previously described in Grainger et al., *Nat. Med.*, 1, 1067 (1995). The cholesterol in fractions 0-9 was assumed to be VLDL, in fractions 10-19 to be LDL, and in fractions 20-30 to be HDL, in accordance with the elution positions of the major apolipoproteins. Lipoprotein concentrations are reported as mM cholesterol. For cell cultures studies, the lipoprotein fraction was subjected to extensive dialysis against serum-free DMEM, and the amount of TGF-beta was measured in the lipoprotein fraction and in the plasma protein fractions after treatment with acid/urea, using the Quantikine ELISA (R&D Systems) in accordance with the manufacturer's instructions.

In some individuals (7/10), TGF-beta was detected in the lipoprotein fraction as well as the lipoprotein deficient plasma fraction. The proportion of the TGF-beta associated with lipoprotein varied from <1% to 39% with a mean of 16%. Thus, plasma TGF-beta, unlike most other plasma proteins, can associate with lipoprotein particles.

TABLE I

Individual	Age (yrs)	Sex	% associated TGF-beta	VLDL	LDL	HDL
A	44	M	27	0.9	3.1	0.8
B	28	M	<1	0.5	2.8	1.1
C	41	F	24	1.1	4.7	0.7
D	33	M	<1	0.6	3.4	0.8
E	28	M	7	0.3	3.0	0.9
F	31	F	19	1.1	2.6	1.0
G	22	M	11	0.8	3.6	0.9
H	49	M	39	1.5	3.3	1.0
I	47	M	<1	0.8	3.7	0.8
J	29	M	9	0.9	3.1	1.0
K	36	M	78	4.6	3.3	0.9
L	27	M	96	1.1	3.8	1.1

To determine whether the TGF-beta associated with lipoprotein particles was able to bind to the type II TGF-beta signaling receptor and exert biological activity in vitro, the binding of recombinant TGF-beta to R2X was measured in the absence and presence of increasing concentrations of lipoprotein purified from the plasma of an individual with <1 ng/ml TGF-beta in plasma (individual I, Table I). If the lipoprotein-associated fraction of TGF-beta is unavailable for binding, lipoproteins prepared from an individual with a very low plasma concentration of TGF-beta would be

expected to reduce the binding of recombinant active TGF-beta to its receptors. The half maximal ( $K_a$ ) binding of recombinant TGF-beta to the recombinant extracellular domain of the type II TGF-beta receptor was previously determined to be 1753 ng/ml (R2X; Grainger et al., *Nature*, 270, 460 (1994); Grainger et al., *Clin. Chim. Acta*, 235, 11 (1995)).

To measure the binding of TGF-beta to its receptor, the recombinant extracellular domain of the type II TGF-beta receptor (R2X), was coated onto ELISA plates (1  $\mu$ g/well, MaxiSorp plates, Gibco BRL). Wells were washed 3 times quickly in TBS and blocked with TBS containing 3% bovine serum albumin (BSA, fatty-acid free; Sigma) for 30 minutes. A standard curve of recombinant active TGF-beta (1.5 ng/ml to 100 ng/ml recombinant active TGF-beta in two fold serial dilutions; R&D Systems) was prepared in TBS +0.1% BSA and in TBS +0.1% BSA additionally containing dialyzed lipoprotein at various concentrations. The standard curves were incubated in the wells containing R2X for 2 hours. The amount of bound TGF-beta was detected with antibody BDA5 (R&D Systems) as previously described by Grainger et al., *Clin. Chim. Acta*, 235, 11 (1995). Briefly, after three quick washes with TBS, the wells were incubated with TGF-beta detection antibody at 1  $\mu$ g/ml in TBS +3% BSA (50  $\mu$ l/well) for 1 hour. After a further three washes in TBS, the wells were incubated with anti-rabbit IgG conjugated to horseradish peroxidase (A-6154; Sigma) at 1:5000 dilution in TBS +3% BSA for 30 minutes. The wells were washed 3 times with TBS and visualized using K-Blue Substrate (Elisa Technologies) for 20 minutes. All incubations were performed at room temperature with shaking (~300 rpm).

The presence of lipoprotein caused a dose-dependent increase in the apparent  $K_a$  for TGF-beta binding to R2X to a maximal value of 425 ng/ml when lipoprotein equivalent to 3 mM total cholesterol was added (FIG. 2A; values are the mean standard error of triplicate determinations). The concentration of lipoprotein (measured as total cholesterol) which half-maximally increased the apparent  $K_a$  was approximately 1 mM. Thus, TGF-beta which is associated with lipoprotein particles has a lower affinity for the type II TGF-beta receptor, or, if the TGF-beta is in equilibrium between the lipoprotein and aqueous phases, is unable to bind to the TGF-beta receptors.

It has previously been shown that TGF-beta inhibits the proliferation of mink lung epithelial (MvLu) cells in culture. Recombinant active TGF-beta was added to MvLu cells (passage 59-63 from the ATCC) which were growing in DMEM +10% fetal calf serum) and the concentration of recombinant TGF-beta required to half-maximally inhibit MvLu cells (reported as MvLu cell  $ED_{50}$ ) was measured as previously described (Danilow et al., *J. Cell Physiol.*, 138, 79 (1989); Kirschenlohr et al., *Am. J. Physiol.*, 265, C571 (1993). Proliferation of MvLu cells was half-maximally inhibited by recombinant active TGF-beta with an  $ID_{50}$  of 0.12 $\pm$ 0.04 ng/ml (n=6) (FIG. 2B). Addition of lipoprotein purified from the plasma of individual I caused a dose-dependent increase in the  $ID_{50}$  of TGF-beta. The  $ID_{50}$  was maximal at 0.52 $\pm$ 0.08 ng/ml when 3 mM total cholesterol was added. The concentration of lipoprotein which half-maximally increased the  $ID_{50}$  was approximately 0.8 mM. Therefore, TGF-beta associated with lipoprotein was less active, or inactive, as an inhibitor of MvLu cell proliferation.

Since low levels of TGF-beta activity have been associated with advanced atherosclerosis, individuals with a large proportion of their plasma TGF-beta sequestered into an

inactive lipoprotein-associated pool may be at significantly higher risk of developing the disease. The differences in the proportion of TGF-beta associated with lipoprotein among the individuals studied was therefore investigated further. The different classes of lipoprotein were separated by size using gel filtration chromatography for ten healthy individuals A-J (Table 1) as well as two diabetic individuals with abnormal lipoprotein profiles (individuals K-L, Table 1). The TGF-beta present in the fractions following the gel filtration of the lipoprotein fraction from each of the ten individuals was then determined.

Individual A had a profile of lipoproteins typical of healthy subjects (FIG. 3A) and 27% of the plasma TGF-beta was associated with the lipoprotein fraction. 88% of the lipoprotein-associated TGF-beta eluted with a tightly defined subfraction of the HDL particles, with the smallest size of all the cholesterol-containing lipoprotein particles. The remaining 12% of the lipoprotein-associated TGF-beta was distributed among the VLDL and LDL fractions. This pattern of association of TGF-beta with a subfraction of HDL particles was typical of all the healthy donors tested (>80% of the lipoprotein-associated TGF-beta in a subfraction of HDL), except individual C.

Individual C had little VLDL or chylomicrons but moderately elevated LDL and 24% of the plasma TGF-beta was associated with the lipoprotein pool (FIG. 3B). As with the other individuals the majority (65%) of the TGF-beta was associated with the HDL subfraction. However, this individual had a significant amount of TGF-beta (27%) associated with LDL and the remainder eluted with the VLDL.

Individual K was a diabetic patient with hypertriglyceridaemia, and >50% of the total plasma cholesterol was present in the largest triglyceride-rich lipoprotein particles (FIG. 3C). This individual had 78% of the plasma TGF-beta associated with the lipoprotein pool, but only 20% of this was present in the HDL subfraction. The remaining 80% co-eluted from the gel filtration column with the VLDL and chylomicrons.

Individual L was a diabetic patient with moderately elevated plasma triglyceride and VLDL/chylomicrons and 92% of the plasma TGF-beta associated with the lipoprotein (FIG. 3D). This individual had very little (<5%) of the lipoprotein-associated TGF-beta co-eluting with the HDL particles. Approximately 60% of the TGF-beta co-eluted with the largest triglyceride-rich lipoprotein particles and the remainder with the LDL particles.

Thus, TGF-beta associated with a subfraction of HDL particles which vary very little in size and which are among the smallest cholesterol-containing lipoproteins present in plasma. Additionally, TGF-beta can associate with both the triglyceride-rich LDL and VLDL particles (FIG. 10). Indeed, under conditions where the concentrations of these particles in plasma is elevated, e.g., in diabetic subjects or patients with hypercholesterolaemia or hypertriglyceridaemia, these particles can become the major lipoprotein fraction responsible for binding TGF-beta.

Diabetic individuals, particularly those with poor glucose control, often exhibit elevated plasma concentrations of the triglyceride-rich lipoprotein particles. Such individuals may therefore have an increased fraction of their plasma TGF-beta associated with the lipoprotein pool, since they may have a major fraction of their plasma TGF-beta associated with the triglyceride-rich lipoprotein particles as well as the subfraction of HDL particles.

The proportion of TGF-beta in the lipoprotein fraction for ten diabetic individuals who exhibited poor glucose control

was determined (Haemoglobin  $\pm 1C-8.0$ ). These individuals had moderately elevated total plasma triglyceride levels ( $2.34 \pm 0.70$  mM compared to  $1.43 \pm 0.60$  mM in healthy control donors;  $n=10$ ;  $p<0.07$  Student unpaired t-test), and the proportion of TGF- $\beta$  associated with lipoprotein was markedly increased ( $68 \pm 21\%$  compared to  $16 \pm 11\%$  in healthy control donors; mean  $\pm$  standard deviation;  $n=10$ ;  $p<0.05$  Mann-Whitney unpaired U-test). Therefore, diabetic individuals with poor glucose control have significantly more of the plasma TGF- $\beta$  sequestered into the lipoprotein pool where it is less active or inactive.

#### EXAMPLE II

##### Effect of Dietary Fish Oil on the Association of TGF- $\beta$ with Lipoprotein

To determine whether dietary supplementation with fish oil would reduce the association of plasma TGF- $\beta$  with the lipoprotein fraction, platelet-poor plasma was prepared from 33 donors prior to, and immediately following, four weeks of dietary supplementation with 2.4 g/day fish oil (Wallace et al., *Arterial Thromb. Vasc. Biol.*, 15, 185 (1995)). A further plasma sample was prepared nine weeks after ceasing the supplementation. The fraction of TGF- $\beta$  associated with the lipoprotein pool was determined for each plasma sample.

At the end of the four week supplementation period total plasma triglyceride concentrations were somewhat reduced although total plasma cholesterol was unaffected (FIG. 4, Table 2). Fish oil supplementation also markedly reduced TGF- $\beta$  association with the lipoprotein fraction. The mean proportion of TGF- $\beta$  associated with lipoprotein was reduced from  $19 \pm 10\%$  (range  $<1\%$  to  $62\%$ ) to  $7 \pm 4\%$  (range  $<1\%$  to  $41\%$ ;  $p<0.01$ ; paired Wilcoxon signed-rank test). After a further nine weeks without fish oil supplementation of the diet, triglycerides had returned to baseline and the proportion of TGF- $\beta$  associated with the lipoprotein pool had increased to  $13 \pm 9\%$ , although it had not returned to the baseline.

Consistent with the decrease in the fraction of TGF- $\beta$  sequestered into the inactive lipoprotein-associated pool, the concentration of active TGF- $\beta$  increased by 21% after four weeks of dietary supplementation with 2.4 g/day fish oil. The concentration of active TGF- $\beta$  was still significantly above baseline after a further 9 weeks after dietary supplementation, although the increase was less marked ( $+12\%$ ,  $p<0.05$ ). Thus, increased dietary intake of fish oil reduces the fraction of plasma TGF- $\beta$  sequestered into the lipoprotein pool, and increases the concentration of active TGF- $\beta$  in plasma.

The reduction in sequestration may be due to the alteration of the proportion of different lipoproteins, i.e., fish oil reduces triglyceride rich lipoprotein levels, or by altering the composition and hence sequestering properties of lipoprotein. Thus, fish oil has no effect on the production of latent TGF- $\beta$  or mature TGF- $\beta$  but increases TGF- $\beta$  bioavailability by decreasing the lipoprotein sequestration of the TGF- $\beta$ . Such an effect would likely result in cardioprotection in individuals with adequate production of latent and mature TGF- $\beta$  but limited ability to release TGF- $\beta$  from lipoprotein complexes.

TABLE 2

Time associated (weeks)	Fish oil supplementation	Total triglyceride (mM)	Total cholesterol (mM)	% TGF- $\beta$
0	None	$1.43 \pm 0.43$	$5.1 \pm 1.2$	$19 \pm 10$ $n = 32$
4	2.4 g/day	$1.03 \pm 0.57$	$5.3 \pm 0.9$	$7 \pm 4^*$ $n = 33$
13	None	$1.56 \pm 0.50$	$5.3 \pm 0.8$	$13 \pm 9$ $n = 31$

Table 2. Proportion of TGF- $\beta$  associated with lipid following dietary supplementation with fish oil. Total triglyceride concentration was measured by the glycerol kinase enzymatic method (Sigma Diagnostics). Total cholesterol and % associated TGF- $\beta$  were assayed as described in Example I. Values are mean  $\pm$  standard error. \* $p<0.01$ ; paired Wilcoxon signed-rank test versus baseline.

#### EXAMPLE III

##### Aspirin Increases Circulating TGF- $\beta$ Levels

Aspirin has been suggested to have cardioprotective effects and is now in widespread use by patients diagnosed with coronary atherosclerosis. It has been demonstrated to significantly reduce the incidence of a second myocardial infarction (MI) in individuals who have previously suffered an MI. However, any benefit for the primary prevention of MI has not yet been demonstrated rigorously, although some studies have reported encouraging results.

A number of effects have been suggested to play a role in the cardioprotective benefits associated with chronic use of low-dose aspirin. Aspirin interferes with normal platelet function and increases the blood clotting time, while decreasing the stability of fibrin deposits. Since chronic formation of mural thrombi is thought to be important in the development of atherosclerosis and acute thrombus formation is the main cause of MI, the anti-platelet function of aspirin is thought to be important in mediating its cardioprotective effects. Moreover, since aspirin is a well-documented anti-inflammatory agent and atherosclerosis has an important inflammatory component, the anti-inflammatory action of aspirin could also contribute to cardioprotection.

In a study of 31 individuals with no detectable atherosclerosis by coronary angiography (NCA), the concentration of active plus acid-activatable latent ( $\alpha+1$ ) TGF- $\beta$  in serum was almost two-fold higher in those taking aspirin (300 mg per day for an average of 30 months) than those not. Thus the proportion of TGF- $\beta$  in the active form was not significantly altered, suggesting that aspirin may stimulate production of the latent TGF- $\beta$  precursor rather than stimulating its activation.

Agents associated with elevated circulating TGF- $\beta$  concentration *in vivo* have been shown to stimulate TGF- $\beta$  production by vascular smooth muscle cells (VSMCs) in culture. To determine whether aspirin could stimulate TGF- $\beta$  production by human VSMCs in culture, confluent cultures of human explant-derived VSMCs were subcultured into and grown for 24 hours in the presence of 10% FCS. The medium was then changed and triplicate wells were treated with either aspirin (from a stock solution dissolved in ethanol) or sodium aspirinate at various concentrations. The medium was replaced after 48 hours and after 96 hours the cells were released with trypsin and

counted by haemocytometer. Tamoxifen (5  $\mu$ M) was used as positive control, since it has previously been shown to stimulate TGF- $\beta$  production under similar conditions. Aspirin inhibited the proliferation of human VSMCs with half-maximal inhibition ( $ED_{50}$ ) at  $12 \pm 3$   $\mu$ M ( $n=4$ ), and maximally inhibited proliferation at 50  $\mu$ M when the increase in cell number over 96 hours was inhibited by 33-66% (FIG. 5A). The effects of sodium aspirinate were not distinguishable from the effects of aspirin ( $ED_{50}$  10  $\mu$ M;  $n=2$ ).

To demonstrate that the inhibition of proliferation by aspirin was due to TGF- $\beta$  production, subcultured human VSMCs were treated with 10  $\mu$ M aspirin in the presence and absence of 25  $\mu$ g/ml of a neutralizing antibody to TGF- $\beta$ . The presence of the antibody abolished ( $>95\%$ ;  $n=3$ ) the growth inhibitory effects of aspirin and sodium aspirinate ( $>95\%$ ;  $n=2$ ) (FIG. 5B).

The amount of TGF- $\beta$  present in medium conditioned on VSMCs in the presence and absence of aspirin was measured by ELISA. In the absence of aspirin, only  $1.5 \pm 0.4$  ng/ml TGF- $\beta$  was detected in the medium compared with  $4.9 \pm 1.2$  ng/ml ( $n=3$ ;  $p<0.05$ ; Students unpaired  $t$  test) after 96 hours in the presence of 10  $\mu$ M aspirin. Thus, aspirin, like tamoxifen, stimulates production of TGF- $\beta$  by human VSMCs in culture, although the  $ED_{50}$  for aspirin (12  $\mu$ M) was markedly less potent than for tamoxifen (50 nM).

To evaluate the effect of aspirin administration on TGF- $\beta$  levels in vivo, the level of (+1) TGF- $\beta$  or TGF- $\beta$  activity in the circulation of 42 patients with more than 50% stenoses of all three major coronary arteries (TVD) taking low-dose aspirin relative to individuals with normal coronary arteries (NCA) was determined. Platelet-poor plasma was prepared with minimal ( $<0.1\%$  assessed by PF-4 release) platelet degranulation and active and (+1) TGF- $\beta$  were measured by ELISA.

The individuals in the NCA group had (+1) TGF- $\beta$  and active TGF- $\beta$  levels typical of healthy individuals reported previously. These patients had either taken no aspirin ( $n=19$ ), or 75 mg ( $n=14$ ), 150 mg ( $n=8$ ) or 300 mg ( $n=1$ ) of aspirin per day for an average of 17 months. There was a significant correlation between aspirin dose and (+1) TGF- $\beta$  levels ( $p<0.05$ ; one way analysis of variation) suggesting that aspirin stimulates TGF- $\beta$  production in a dose-dependent manner (FIGS. 6A and 6B). The mean (+1) TGF- $\beta$  level was significantly higher in patients taking 75 mg/day of aspirin ( $+41\%$ ;  $p<0.05$ ), and in patients taking 150 mg/day of aspirin ( $55\%$ ;  $p<0.05$ ). This is consistent with a previous study, where (+1) TGF- $\beta$  levels were elevated by 66% in patients taking 150 mg aspirin per day. TGF- $\beta$  activity was also elevated in NCA individuals taking 150 mg aspirin per day (FIG. 6A), and hence the proportion of TGF- $\beta$  in the active form was not significantly changed. TGF- $\beta$  production was similarly higher in both men and women taking aspirin ( $+47\%$  in men compared to  $44\%$  in women at 150 mg per day; FIG. 6C).

#### EXAMPLE IV

##### Copper Aspirinate is a TGF- $\beta$ Stimulating Agent

One disadvantage of aspirin as a TGF- $\beta$  stimulator is that aspirin is not very potent in human cell culture or in vivo. Therefore, the identification of other TGF- $\beta$  production stimulators which are more potent than aspirin is needed.

Consumption of red wine has been proposed to mediate cardiovascular protection, although the data supporting this

proposal are still debated. To determine whether red wine, as opposed to white wine, can stimulate TGF- $\beta$  production in vitro or in vivo, red wine (Chateaux 1973 from the Bordeaux region) or white wine (German Reissling) was lyophilized and reconstituted in one tenth volume of 5% ethanol in water to produce a 10 fold wine concentrate. Red and white grape juice (Sainsbury's) were treated similarly as controls as they are expected to lack the active components produced during fermentation of the grape skins. Rat vascular smooth muscle cells (rVSMCs) were subcultured into DMEM+10% fetal calf serum, grown for 24 hours then treated with various concentrations of the wines or grape juices. The medium was replaced after 48 hours and after 96 hours, the cells released with trypsin and counted by haemocytometer. Final concentrations of the wine and grape juice on the cells were expressed as a percentage of the concentration of the original wine or grape juice.

Red wine, but not white wine or either grape juice, inhibited VSMC proliferation with an  $ED_{50}$  of 25-33% concentration. At the highest concentration tested (200%) the increase in cell number after 96 hours was inhibited by 46-66% ( $n=3$ ).

To determine whether this inhibition of VSMC proliferation by the red wine concentrate was due to induction of TGF- $\beta$ , cells were incubated with 25% and 100% concentration of red wine in the presence and absence of 10  $\mu$ g/ml of a neutralizing antiserum to TGF- $\beta$ , which has previously been shown to completely abolish the growth inhibitory effects of 10 ng/ml TGF- $\beta$  in VSMC culture. The presence of neutralizing antibody to TGF- $\beta$  completely reversed ( $>95\%$ ) the growth inhibitory effects of the red wine. Thus, red wine induces TGF- $\beta$  production by VSMCs in vitro and this effect is not due to the alcohol component.

To investigate whether red wine might also elevate TGF- $\beta$  levels in vivo, blood samples were drawn from 120 randomly selected individuals in Toulouse, France and serum prepared. Additionally, these subjects completed a questionnaire which included details of their wine consumption. Active plus latent (+1) TGF- $\beta$  and active TGF- $\beta$  levels in these samples were assayed by ELISA as described hereinabove. The mean (+1) TGF- $\beta$  and active TGF- $\beta$  levels were not significantly different from those reported from other random populations.

A weaker correlation was observed between red wine consumption and active TGF- $\beta$  levels. Thus, (+1) TGF- $\beta$  was almost two fold higher in the group of individuals drinking more than 1 standard deviation above the mean red wine consumption than those more than 1 standard deviation below the mean. Although there was also a significant correlation between total alcohol consumption and (+1) TGF- $\beta$  levels, this may result from the large fraction of total alcohol consumed which is taken as red wine in this population. There was no correlation between white wine, beer or spirit consumption and either (+1) TGF- $\beta$  or active TGF- $\beta$  levels. Thus, it is very likely that increased wine consumption is associated with elevated TGF- $\beta$  levels in the circulation.

When the red wine consumed was divided by the region of origin, four regions were significantly represented. However, of these, only individuals drinking wine originally from Bordeaux showed a statistically significant correlation with (+1) TGF- $\beta$  levels. This may be a consequence of the reduced numbers of individuals in each group, or alternatively suggests that only wines of Bordeaux origin stimulate TGF- $\beta$  production or increase TGF- $\beta$  levels more potently than wines of other origins.

Red wines, but not white wines, have been shown to contain various salicylate components which are produced during fermentation of the grape skins. To determine whether a salicylate component of red wine was correlated to TGF-beta activity, cultured (rat or human) VSMCs were exposed to red wine in the presence of various concentrations of a neutralizing antibody raised against sodium salicylate coupled to keyhole limpet hemocyanin as a carrier protein. The anti-salicylate antibody reversed the growth inhibitory activity of Bordeaux red wine with an  $ED_{50}$  of 15  $\mu$ g/ml. At concentrations of 33  $\mu$ g/ml and above, maximal reversal of the growth inhibition was achieved, i.e., approximately 70% of the growth inhibitory activity was reversed. The majority of the TGF-beta stimulating activity in Bordeaux red wine is therefore due to the presence of salicylate-like compounds.

Given the likely concentration of salicylate in this red wine, based on previous studies, and the  $ED_{50}$  for aspirin inducing TGF-beta in VSMC culture, the presence of salicylate alone cannot explain the observed effects. One possible resolution of this paradox would be the presence of salicylate-like compounds in red wine which stimulate TGF-beta more potently than acetyl salicylate. One such derivative, reported to have more potent effects than aspirin is the transition metal complex copper II (acetyl salicylate)<sub>2</sub>. The  $ED_{50}$  for TGF-beta production of the complex (Cu Aspirinate) was determined in cultured rat and human VSMCs. Cu aspirinate was almost two orders of magnitude more potent than aspirin at stimulating TGF-beta ( $ED_{50}$  on human cells 200 nM for Cu aspirinate versus 10  $\mu$ M for aspirin). It is likely that there is sufficient Cu aspirinate in red wine, and particularly in red wines of Bordeaux origin which are especially rich in copper, to account for most if not all of the TGF-beta stimulating activity associated with red wine.

Thus, copper aspirinate complex is believed to be the active TGF-beta stimulating agent in red wine and is a potent TGF-beta production stimulating agent in vitro and in vivo.

#### EXAMPLE V

##### TGF-beta Levels in Thromboxin Treated Patients

To investigate whether TGF-beta levels are elevated after TMX administration, fifteen patients with stable angina and angiographically defined triple vessel disease took TMX daily for ten days at a dose similar to that generally used for breast cancer therapy. Patients with triple vessel disease (TVD) were defined as individuals having at least 50% stenosis of all three coronary arteries by angiography, which was confirmed by two independent observers. All had stable angina, with no myocardial infarction in the previous three months. Patients with unstable angina, poor left ventricular function, ventricular hypertrophy or diabetes were excluded.

Blood samples were taken and plasma prepared before and during the treatment period, and these samples were analyzed for TGF-beta, Lp(a), PAI-1 and lipoprotein profiles. Patients were asked to fast overnight prior to samples of blood being drawn between 9 a.m. and 10 a.m. the following morning. Blood samples were drawn by venipuncture of the antecubital vein with no tourniquet applied using a 21 gauge butterfly needle. Half the blood was allowed to clot for 2 hours at room temperature in polypropylene tubes. The clot was spun down (1,500g; 15 minutes) and aliquots of the serum was stored at -100° C. The remaining blood was dispensed into Diaube H tubes (Diagnostics Stago) and cooled on ice for 15 minutes. Blood

cells and platelets were spun down (6,000g; 30 minutes) and the middle third of the supernatant was taken, carefully avoiding disturbing the pellet. This platelet-poor plasma was stored in aliquots at -100° C until assayed. For all samples, assay for PF-4 demonstrated that less than 0.02% of the platelets had degenerated during plasma preparation.

Active plus acid-activatable latent ((+1) TGF-beta levels were assayed in platelet-poor plasma using seven different assay methods. There has been debate in the literature regarding the most appropriate way to measure ((+1) TGF-beta, so to avoid difficulties specific to any particular measurement method all the available methods which have been described in the literature were used. The seven methods are:

- (A) A sandwich ELISA using BDA19 and BDA5 (R&D Systems) antibodies with no activation step required.
- (B) The Quantikine TGF-beta1 ELISA kit (R&D Systems) using acid/urea as the activation buffer in accordance with the manufacturer's instructions
- (C) The Quantikine TGF-beta1 ELISA kit (R&D Systems) using acid alone as the activation buffer
- (D) The BioTrak TGF-beta1 ELISA kit (Amersham International) using acid/urea as the activation buffer
- (E) The BioTrak TGF-beta1 ELISA kit (Amersham International) using acid alone as the activation buffer in accordance with the manufacturer's instructions
- (F) The TGF-beta1 ELISA kit (Cemzyme Diagnostics) using acid/urea as the activation buffer in accordance with the manufacturer's instructions
- (G) The TGF-beta1 ELISA kit (Promega Corporation) using acid alone as the activation buffer in accordance with the manufacturer's instructions

Replicate aliquots of plasma taken from the same individuals at the same time were assayed by all seven methods.

The partitioning of TGF-beta between the lipoproteins and plasma proteins was analyzed by separating the lipoprotein fraction (d=1.215 g/cm<sup>3</sup>) from the plasma proteins by density ultracentrifugation as described hereinabove. TGF-beta levels were assayed in both fractions using Quantikine ELISA kit, following release and activation of TGF-beta with acetic acid and urea in accordance with the manufacturer's instructions. None of the three TGF-beta ELISAs used here detected TGF-beta in the lipoprotein fraction without prior extraction/activation with acetic acid/urea.

Total plasma triglyceride, total plasma cholesterol, HDL-cholesterol, LDL-cholesterol and VLDL-cholesterol were routinely assayed in all patients. Liver function tests (aspartate transaminase and lactate dehydrogenase) were also performed on samples prior to dosing with TMX and at the end of the study by a clinical biochemistry laboratory. Plasma PAI-1 was assayed using an ELISA (American Diagnostics) which recognizes active endothelial PAI-1 as well as inactive PAI-1 complexes. Lipoprotein(a) was assayed by an ELISA for apolipoprotein(a) (Immu-no) which showed no detectable cross reactivity with related proteins such as plasminogen. Platelet factor-4 (PF4) and  $\beta$ -thromboglobulin (PTG) were assayed using specific ELISAs (Diagnostics Stago).

Many of the parameters studied would not be expected to show a normal distribution in the population of TVD patients. Consequently, all comparisons are made with the baseline (day 0) values using the paired Wilcoxon signed-rank test. A p value of 0.05 or less was taken to indicate statistical significance.

TMX [Nolvadex<sup>TM</sup> (tamoxifen citrate), Zeneca Ltd., Macclesfield, UK] at a dose of 40 mg was taken each

morning, before breakfast, for 10 days. Before TMX treatment the mean ( $\pm$ 1) TGF- $\beta$  in plasma was  $6.2 \pm 1.3$  ng/ml.

During treatment with TMX, there was a trend of increasing concentration of ( $\pm$ 1) TGF- $\beta$  irrespective of the assay method used (Table 3). Each of the assay methods were standardized against different TGF- $\beta$  standard curves and gave significantly different median levels of ( $\pm$ 1) TGF- $\beta$  in the population at baseline. However, by day 10 there was a median increase of 59% in ( $\pm$ 1) TGF- $\beta$ . This increase was statistically significant for all the assay methodologies used, except for (G). This kit did not perform well, detecting much lower levels of ( $\pm$ 1) TGF- $\beta$  at both baseline and after treatment than all the other methods (A) to (F).

Therefore, during treatment of men with TMX there is a statistically significant increase in the amount of ( $\pm$ 1) TGF- $\beta$  in plasma, by 10 days after treatment is commenced. This increase is detected irrespective of the methodology used to measure ( $\pm$ 1) TGF- $\beta$ .

TABLE 3

	Day 0	Day 10
Age (yr)	62.2 $\pm$ 3.5	
Total plasma cholesterol (mM)	6.31 $\pm$ 0.28	5.95 $\pm$ 0.29*
VLDL-cholesterol (mM)	1.03 $\pm$ 0.34	0.94 $\pm$ 0.13*
LDL-cholesterol (mM)	4.48 $\pm$ 0.27	4.16 $\pm$ 0.25
HDL-cholesterol (mM)	0.78 $\pm$ 0.03	0.77 $\pm$ 0.04
Total plasma triglycerides (mM)	2.79 $\pm$ 0.44	2.28 $\pm$ 0.35
Plasma ( $\pm$ 1) TGF- $\beta$ (ng/ml)		
Method (A)	6.2 $\pm$ 1.3	7.7 $\pm$ 1.5* (+24%)
Method (B)	0.7 $\pm$ 0.1	1.2 $\pm$ 0.2* (+71%)
Method (C)	0.25 $\pm$ 0.07	0.62 $\pm$ 0.07** (+148%)
Method (D)	2.0 $\pm$ 0.1*	2.4 $\pm$ 0.1* (+20%)
Method (E)	1.7 $\pm$ 0.1	2.3 $\pm$ 0.1** (+35%)
Method (F)	3.9 $\pm$ 0.1	5.2 $\pm$ 0.3** (+33%)
Method (G)	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1 (+100%)
Lipoprotein(a) (mg/dl)	61.3 $\pm$ 13.8	42.4 $\pm$ 9.2**
Plasma PAI-1 antigen (ng/ml)	29.3 $\pm$ 6.4	35.0 $\pm$ 5.4*

All values are mean  $\pm$  standard error for 15 patients. Comparisons between baseline (day 0) and values after TMX treatment (day 10) were made using the paired Wilcoxon signed-ranks test.

\*,  $p < 0.05$ .

\*\*,  $p < 0.01$ .

These patients had a high average level of Lp(a) (mean  $61 \pm 10$  mg/dl at baseline), consistent with their coronary artery status. The plasma concentration of Lp(a) was decreased by 27% ( $p < 0.05$  compared to baseline) by day 3 of TMX therapy. By day 10, the Lp(a) concentration was reduced by 31% compared to baseline ( $p < 0.02$ ; FIG. 8A).

Another cardiovascular risk factor which has been shown to influence TGF- $\beta$  activity is the lipoprotein profile, since TGF- $\beta$  can be sequestered into lipoprotein particles where it is biologically inactive. TMX has been reported to decrease plasma cholesterol and to increase the fraction of cholesterol in HDL particles. Consistent with these reports, total plasma cholesterol was decreased by 6% below baseline ( $p = 0.04$ ) after 10 days of TMX therapy. In addition, cholesterol in the VLDL fraction was reduced (18% below baseline;  $p = 0.04$ ) but the concentration of LDL-cholesterol and HDL-cholesterol were both unchanged (Table 3). Total plasma triglyceride concentration was 18% lower after 10 days of TMX treatment, but the change was not statistically significant ( $p = 0.22$ ).

Since TMX significantly altered the lipoprotein profile of the patients, the proportion of the plasma TGF- $\beta$

associated with lipoprotein was measured. The lipoproteins were separated from the plasma proteins by density gradient ultracentrifugation. In order to detect the TGF- $\beta$  in the lipoprotein fraction, the Quantikine ELISA was used following release and activation of any TGF- $\beta$  in both the lipoprotein fraction and the plasma protein fraction. At baseline 34  $\pm$  4% of the TGF- $\beta$  was lipoprotein-associated and hence biologically inactive, but this was reduced to 25  $\pm$  3% ( $p < 0.01$ ) after 10 days of TMX therapy (FIG. 8C).

The data show that TMX (40 mg per day) elevates the plasma concentration of TGF- $\beta$  in men with severe coronary atherosclerosis. This increase was seen irrespective of which of the seven different methodologies were employed to measure ( $\pm$ 1) TGF- $\beta$ . Consistent with studies in cell culture and in mice, TMX elevates the amount of ( $\pm$ 1) TGF- $\beta$ , suggesting that the elevation may have resulted from increased synthesis of latent precursor complexes. In rat and human smooth muscle cell culture, TMX increases TGF- $\beta$  production by increasing the amount of TGF- $\beta$  mRNA. In other cell types TMX increases the translational efficiency of TGF- $\beta$  mRNA and hence increases production of the latent precursor protein. Irrespective of the mechanism, we observe increased levels of TGF- $\beta$  in men with atherosclerosis, corresponding to the increases seen in animal models of atherosclerosis when TMX significantly reduces lipid lesion formation, irrespective of the genetic predisposition to lesion formation (FIG. 9).

## EXAMPLE VI

Combination Therapies to Elevate the Level of TGF- $\beta$ 

Another disadvantage of aspirin as a cardiovascular agent, besides the fact that it is not a very potent TGF- $\beta$  elevating agent, is that it appears to be a pure stimulator of the latent form of TGF- $\beta$ . As a result, under conditions where TGF- $\beta$  activation or release is not occurring, or is occurring to a reduced extent, e.g., when PAI-1 inhibits activation or lipoproteins sequester TGF- $\beta$ , the supply of latent TGF- $\beta$  precursors may not be limiting for the generation of the active forms. This disadvantage can be overcome by combination therapy. Thus, the identification of agents that increase the level of mature and/or active TGF- $\beta$ , can be useful in combination therapies with aspirin or with other agents that are more potent stimulators of the latent form of TGF- $\beta$ , such as copper aspartate.

To determine the efficacy of combination therapy, and to provide evidence for synergism between aspirin and fish oil, 8-week-old female apoE knockout mice were fed aspirin or fish oil, or both, to assess the cardioprotective effects of modulating different components of the TGF- $\beta$  pathway.

Group A mice ( $n = 10$ ) were sacrificed at day 0. Group B mice ( $n = 10$ ) were fed normal chow. Group C mice ( $n = 10$ ) were fed normal chow and about 3 mg/kg/day aspirin dissolved in water (15  $\mu$ g/ml aspirin). Group D mice ( $n = 10$ ) were fed chow containing 33 mg/kg/day fish oil (200  $\mu$ g Pulse cod liver oil/g food, Seven Seas Ltd., which contains 0.9 g eicosapentaenoic acid (EPA), and 0.3 g docosahexaenoic acid (DHA)) and 3 mg/kg/day aspirin dissolved in water. Group E mice ( $n = 10$ ) were fed chow containing 33 mg/kg/day fish oil. Group F mice ( $n = 10$ ) were fed chow containing Zocor (simvastatin; Zocor tablets, Merck, Sharpe & Dohme) at 400  $\mu$ g/kg/day (2  $\mu$ g/g food). Simvastatin is an inhibitor of the enzyme HMG-CoA reductase, the committed step in cholesterol biosynthesis. As a result, it has been shown to reduce the total plasma cholesterol concentration in man and in particular the concentration of cholesterol in

the more triglyceride-rich particles (VLDL and LDL). If alterations in the lipid profile are responsible for the suppression of lesion formation previously observed with TMX, then simvastatin should reduce lesion formation.

Groups B-F were fed these regimens for 87 days. All mice were weighed daily for the first week and weekly thereafter. Food and water consumption over a 24 hour period were measured daily for the first 7 days and weekly thereafter. There was no significant difference in weight, food intake or water consumption in any of the groups throughout the study.

After 87 days, mice in groups B-F were fasted overnight and then sacrificed. Serum, heart, lungs and aorta samples were collected at the time of sacrifice. The heart, lungs and aorta were removed from each mouse and rinsed in PBS, dabbed dry on tissue and embedded in Cryo-M-bed embedding medium (Bright Instruments, Huntington, U.K.) before snap freezing in liquid nitrogen. Frozen sections (4  $\mu$ m thickness) of the aortic sinus region were prepared from the heart/lung/aorta blocks according to the sectioning strategy of Paigen et al. (*Atherosclerosis*, 10, 316 (1990)). Sections on 5% gelatin-coated slides were stained for neutral lipid by the Oil Red O technique and counter-stained with fast green (Grainger et al., *Nature Med.*, 1, 1067 (1995)).

The development of lipid-filled vascular lesions was determined by the quantitation of oil red O staining for neutral lipid deposited in the aortic sinus region. The area of lipid accumulation was measured using a calibrated microscope eye-piece, such that lipid droplets <50  $\mu$ m<sup>2</sup> were ignored and contiguous regions of lipid staining >500  $\mu$ m<sup>2</sup> in area were classified as lesions. The area staining for neutral lipid increased from 10,765 $\pm$ 978 to 27,175 $\pm$ 1040  $\mu$ m<sup>2</sup>/mouse over the three months of the experiment for mice fed a normal mouse chow diet, as seen in previous studies of spontaneous lesion development in apoE knockout mice. Treatment with aspirin alone did not affect lesion development over the same 3 month period (Table 4). Treatment with fish oil alone reduced lesion development slightly, although the variation in the area of lesions between animals within a group was too large for the effect to be statistically significant (-8%;  $p=0.11$ ; Mann-Whitney U test).

In contrast, treatment with aspirin plus fish oil resulted in a significant reduction in lesion formation (-22%;  $p=0.01$ ; Mann-Whitney U test), suggesting that aspirin and fish oil act synergistically to reduce lipid lesion formation. Treatment with simvastatin, however, did not significantly reduce lipid lesion formation in apoE knockout mice. The area staining for neutral lipid deposition was lower than in untreated mice (-7%;  $p=0.33$ ; Mann-Whitney U test), but as with mice treated with fish oil alone, this decrease was not statistically significant.

Treatment with aspirin and fish oil, alone or in combination, also resulted in a marked change in lesion morphology. The area of cellular intima that formed was reduced, most markedly in the group which received the combination of aspirin and fish oil, and the lipid staining was confined to a region close to the internal elastic lamina. As a result, the lesions coalesced and the number of separate lesions decreased even where the total area staining for lipid accumulation was unchanged.

TABLE 4

Group	Treatment	Lesion Area ( $\mu$ m <sup>2</sup> /mouse)	Number of Lesions
A	control, day 0	10,765 $\pm$ 978	3.4 $\pm$ 0.2
B	control, day 88	27,175 $\pm$ 1040	10.2 $\pm$ 0.7
C	aspirin	27,512 $\pm$ 974	6.6 $\pm$ 0.2**
D	aspirin + fish oil	23,587 $\pm$ 898**	5.5 $\pm$ 0.4**
E	fish oil	25,871 $\pm$ 1356	6.9 $\pm$ 0.3**
F	Zinc	25,777 $\pm$ 1368	8.1 $\pm$ 0.4*

\* $p < 0.05$ .\*\* $p < 0.05$  Mann-Whitney U test

The amount of active plus latent and active TGF-beta in the vessel wall, and the amount of active TGF-beta (ng/ml) in the plasma of these mice was also determined (Table 5), by methodologies described in Examples 4 and 7 of copending U.S. application Ser. No. 08/478,936, filed Jun. 7, 1995, which is incorporated by reference herein.

The neighboring sections to those stained for neutral lipid with Oil Red O described above were taken onto slides coated with poly-L-lysine (0.1%; Sigma) and fixed in ice-cold acetone for 90 seconds, air-dried and stored at -20°C until assayed. Active plus acid-activatable latent (a+1) TGF-beta was measured by quantitative immunofluorescence microscopy using specific primary antibodies (BDA 19, AB-100-NA; R&D Systems), see Mosedale et al., *Histochem. Cytochem.*, 44, 1043 (1996), the disclosure of which is incorporated by reference herein. Active TGF-beta was measured by quantitative immunofluorescence microscopy using the recombinant extracellular domain of the type II TGF-beta receptor (R2X) labeled with fluorescein.

The data in Table 5 show that active plus latent TGF-beta levels were significantly elevated in the vessel wall of mice having diets supplemented with aspirin (+36%;  $p=0.01$ ;  $n=10$ , Mann-Whitney U test) relative to control mice. The amount of active TGF-beta was not significantly affected by aspirin therapy (-6%;  $p=NS$ ), suggesting that the additional latent complexes were not efficiently activated in the vessel wall of apoE knockout mice. In contrast, fish oil treatment for three months did not affect (a+1) TGF-beta (+5%;  $p=NS$ ) but elevated active TGF-beta to a small extent (+17%;  $p=0.05$ ;  $n=10$ ; Mann-Whitney U test). These results suggest that aspirin stimulates production of latent TGF-beta complexes, while fish oil increases the proportion of TGF-beta available in the active form, i.e., for receptor binding.

TABLE 5

Group	Active + Latent TGF-beta in Vessel Wall	Active TGF-beta in Vessel Wall
A	54 $\pm$ 4	36 $\pm$ 2
B	42 $\pm$ 4	18 $\pm$ 2
C	57 $\pm$ 3**	17 $\pm$ 2
D	63 $\pm$ 6**	24 $\pm$ 3**
E	44 $\pm$ 4	21 $\pm$ 3*
F	44 $\pm$ 5	20 $\pm$ 3

\* $p < 0.05$ .\*\* $p < 0.05$  Mann-Whitney U test

Group D mice, which were treated with both aspirin and fish oil had significantly elevated levels of both (a+1) TGF-beta (+50%) and active TGF-beta (+33%) in the vessel wall compared with the control mice. The synergism of the effects of these drugs on the amount of active TGF-beta in the vessel wall is consistent with the proposed different mechanisms of action for the two drugs. Taken with the

results shown in Table 4, an increase in level of active TGF-beta in apo(E)-/- mice correlates with a decrease in lesion number and area.

Simvastatin treated mice (Group F) showed no difference in the amounts of (a+1) TGF-beta and active TGF-beta in the vessel wall. Thus, in the apoE knockout mouse, any beneficial effects of simvastatin are unlikely to be attributed to elevation of TGF-beta activity. Similarly, any beneficial effects of the aspirin plus fish oil therapy on the lipoprotein profile are unlikely to have contributed to the therapeutic reduction in lesion area by this therapy.

In summary, agents that elevate TGF-beta activity in the vessel wall reduce or inhibit lipid lesion development in mouse aorta, while agents which do not affect TGF-beta activity are ineffective (Table 6). Furthermore, the statistical correlation between the magnitude of TGF-beta activity elevation and lesion area inhibition is very marked, suggesting that the greater increase in vessel wall TGF-beta activity which is achieved, the greater the inhibition of lesion development. This correlation provides powerful evidence supporting the role of TGF-beta activity in mediating the cardioprotective activity of both tamoxifen, and aspirin and fish oil.

TABLE 6

Treatment	% Lesion Suppression	Fold increase in Active TGF-beta
Nose	0	1.00
Aspirin	-2	0.95
Fish Oil	8	1.1
Aspirin and Fish Oil	17*	1.3*
TMX	99*	1.9*

\*Statistically significant,  $p < 0.001$ , Pearson's R correlation,  $r = 0.73$

The effects of these treatments on the cellular changes associated with lesion formation, marked by the accumulation of osteopontin and loss of smooth muscle  $\alpha$ -actin (SM- $\alpha$ -actin) in the vessel wall, which has been shown to be characteristic of lesion formation in man and animal models of atherosclerosis, was also examined. SM- $\alpha$ -actin and osteopontin were measured by quantitative immunofluorescence microscopy using specific primary antibodies A-6125 (Sigma) and MBP111B (NIH Developmental Studies Hybridoma Bank), respectively.

As lipid lesions developed over 3 months on a normal mouse chow diet, staining for SM- $\alpha$ -actin decreased (-36%;  $p < 0.01$ ;  $n=10$ ; Mann-Whitney U test), while staining for osteopontin increased (+150%;  $p < 0.01$ ;  $n=10$ ; Mann-Whitney U test). Of the treatments used in this study, only the combination of fish oil and aspirin abolished the loss of SM- $\alpha$ -actin and the accumulation of osteopontin (Table 7), consistent with the observation that this was the only treatment regimen which significantly reduced lipid accumulation into the vessel wall. The increase in SM- $\alpha$ -actin in mice treated with fish oil or fish oil plus aspirin is consistent with the observed increase in SM- $\alpha$ -actin in apo(E)-/- mice treated with TMX.

TABLE 7

Group	SM- $\alpha$ -Actin	Osteopontin
A	157 $\pm$ 22	32 $\pm$ 7
B	101 $\pm$ 12	80 $\pm$ 9
C	87 $\pm$ 9*	84 $\pm$ 7
D	194 $\pm$ 18**	35 $\pm$ 8**

TABLE 7-continued

Group	SM- $\alpha$ -Actin	Osteopontin
E	122 $\pm$ 17	67 $\pm$ 10*
F	114 $\pm$ 19	73 $\pm$ 8

\* $p < 0.01$ .

\*\* $p < 0.05$  Mann-Whitney U test

TABLE 8

	Active & Latent TGF-beta	Active TGF-beta	SM- $\alpha$ -Actin	Oil Red O
Active & latent TGF-beta		$r = 0.58^{**}$	$r = 0.67^{***}$	$r = -0.06$
Active TGF-beta			$r = 0.76^{***}$	$r = -0.32^{*}$
SM- $\alpha$ -actin				$r = -0.13$
Oil Red O				

\* $p < 0.01$

\*\* $p < 0.001$

\*\*\* $p < 0.0001$

As shown by the correlations of data from Groups A-F summarized in Table 8, an increase in the level of active TGF-beta correlates with an increase in SM- $\alpha$ -actin expression. This result is consistent with the hypothesis that active TGF-beta regulates smooth muscle cell differentiation in vivo. Moreover, active TGF-beta, but not active plus latent TGF-beta, negatively correlates with lesion area, suggesting that active TGF-beta protects against lesion development.

The effect of each treatment on the lipid profile of each group of mice was determined by measuring the cholesterol and triglyceride. Blood from a terminal bleed was collected in a polypropylene tube, allowed to clot at room temperature for 2 hours and then spun (1,000xg; 5 minutes). The serum supernatant was aliquoted and stored at -20°C until assayed. Total cholesterol and total triglycerides were determined for each mouse using the cholesterol oxidase and glycerol kinase UV end-point enzymatic methods respectively (Sigma Diagnostics). For determination of the lipoprotein profile, 100  $\mu$ l of serum from each group and the group was pooled (a total of 1 ml serum for each group) and the lipoprotein fraction was separated by density gradient ultracentrifugation. The lipoprotein fraction was then further separated by gel filtration FPLC chromatography on a Sepharose 6B column, and the elution positions of the lipoprotein particles were detected by measuring cholesterol (by the cholesterol oxidase enzymatic method) in each fraction. VLDL particles eluted in fractions 1-10, LDL in fractions 11-20 and HDL in fractions after 20.

Treatment of the mice with aspirin for three months had no effect on total plasma cholesterol or on the lipoprotein profile (Table 8). Mice treated with diets containing fish oil (with or without aspirin) had similar total plasma cholesterol and triglyceride concentrations to control mice, although there was a small reduction in the concentration of both VLDL-cholesterol (-16%) and LDL-cholesterol (-12%) and an increase in HDL-cholesterol (+10%). Consistent with the effects of dietary supplementation with fish oil in man, a decrease in cholesterol, primarily in the VLDL fraction, in apoE knockout mice treated with fish oil was observed.

There was a significant reduction in total plasma cholesterol in apoE knockout mice treated with simvastatin (-27%;  $p < 0.01$ ;  $n=10$ ; Student's unpaired t-test). Much of this reduction occurred in the VLDL fraction (-14%) and LDL fraction (-41%), with an increase in HDL-cholesterol. In

contrast, TMX lowered VLDL by seven fold and is a much more powerful lipid-lowering agent in the apo(E)- mouse than simvastatin. Simvastatin also caused a significant reduction in total plasma triglyceride concentration (~12%). Consequently, for all lipoprotein parameters measured, simvastatin had a significantly more beneficial effect than aspirin and fish oil either alone or in combination.

TABLE 9

	Group A	Group B	Group C	Group D	Group E	Group F
Total cholesterol (mg/dl)	n.d.	306 ± 31	282 ± 28	273 ± 19	266 ± 25	224 ± 29**
Total triglyceride (mg/dl)	n.d.	302 ± 28	320 ± 19	308 ± 25	296 ± 33	266 ± 14**
VLDL-cholesterol (mg/dl)	n.d.	184	179	157	151	158
LDL-cholesterol (mg/dl)	n.d.	92	89	91	88	54
IDL-cholesterol (mg/dl)	n.d.	30	26	32	33	35

\*\*p < 0.001; Mann-Whitney U test

n.d. = not determined.

A single measurement of the lipoprotein profile was made on blood pooled from all the mice in the Group.

Moreover, the percentage of TGF-beta sequestered in VLDL in Groups B-F and C57B16 mice, which were fed a high fat diet, showed that lipid sequestration of active TGF-beta was not a major mechanism of the inhibition of TGF-beta activity in apo(E)- mice.

In summary, aspirin and fish oil act synergistically to reduce acute lipid lesion development in a mouse model of severe atherosclerosis. While aspirin or fish oil alone reduced the development of vascular lipid lesions in apoE knockout mice over a three month treatment period, a combination of aspirin plus fish oil therapy resulted in a greater reduction (22%) in lesion formation. If low dose aspirin therapy and dietary supplementation with fish oil differ in their mechanism of action, then their cardioprotective effects would be expected to be additive. However, the results described hereinabove provide evidence that the combination of aspirin and fish oil exerts a markedly synergistic effect. Thus, a combination of low dose aspirin and fish oil therapy can be very useful in cardiovascular disease prevention. Moreover, because fish oil is not a very effective VLDL lowering agent, more powerful VLDL lowering agents, such as TMX, can be employed in combination therapies with aspirin, aspirinate salts to result in more beneficial cardiovascular effects.

Consistent with data in humans, aspirin increases the level of latent TGF-beta, but not the amount of active TGF-beta, in the vessel wall of apo(E)- mice. Also consistent with data in humans, fish oil lowers VLDL, which results in lower levels of PAI-1 and an increase in the levels of active TGF-beta which are available for TGF-beta receptor binding.

Previously, tamoxifen treatment has been demonstrated to elevate TGF-beta activity and suppress lipid lesion formation in several transgenic mouse models of atherosclerosis (Grainger et al.). However, tamoxifen has a variety of other effects, including reducing total plasma cholesterol and inducing some weight loss, which may have contributed to the observed reduction in lesion development. As a result, it could not be concluded that elevating TGF-beta activity reduced lesion formation. In contrast, the study described hereinabove employed agents which elevate TGF-beta activity and which do not affect body weight and have much smaller effects on lipoprotein metabolism. Furthermore, simvastatin, which has a larger beneficial effect on the

lipoprotein profile than the other treatments, does not significantly reduce lipid lesion formation. Since there is a significant correlation between increase in TGF-beta activity and decrease in lipid lesion formation for all the therapies (~0.909, p<0.001), it can be concluded that elevation in TGF-beta activity is likely to be involved in the mechanism by which these agents reduce lesion formation in mammals.

## EXAMPLE VII

### Use of Therapeutic Agents of the Invention to Prevent Autoimmune Disorders

The therapeutic agents of the invention are also useful to prevent or treat other indications associated with TGF-beta, e.g., pathologies which result from a pathological inflammatory reaction caused by the recognition of self-antigens ("autoimmune disorders"). Indications associated with pathological inflammation reactions include, but are not limited to, rheumatoid arthritis, multiple sclerosis and late-onset diabetes. The recruitment and activation of both autoreactive T cells and other inflammatory cells to the developing lesion contributes to both the chronic tissue damage and the acute symptoms of autoimmune disorders. Agents which reduce or prevent immune cell recruitment and/or activation may ameliorate both the painful symptoms associated with the disorder and the progressive destruction of the target tissue.

Current treatments for autoimmune disorders include the administration of anti-inflammatory steroids and steroid-mimetic drugs, such as dexamethasone, to reduce recruitment and activation of the immune cells in the developing lesions. These drugs act by binding to the glucocorticoid receptor (GR) which leads to the association of the GR with elements of the NFkB transcription factor complex. When the GR interacts with the NFkB complex, pro-inflammatory cytokines are inhibited. The binding of the steroids to the GR also results in the activation of GR. Activated GR is a nuclear transcription factor. Thus, a set of genes are activated in response to the binding of a steroid or steroid-mimetic drug to GR. This pathway is illustrated in FIG. 12. However, steroids and steroid-mimetic drugs cannot be used chronically to slow the progression of autoimmune diseases because they have an undesirable profile of side effects. Many or all of these side effects result from the direct activation of the GR as a transcription factor.

Thus, agents which modulate the interaction of the estrogen receptor (ER) with the NFkB transcriptional complex ("ER/NFkB modulators") without activating GR are useful to prevent or treat conditions characterized by the recruitment of autoreactive immune cells into tissue and the subsequent damage or destruction of that tissue by chronic inflammation. Preferred ER/NFkB modulators include idoxifene, raloxifene, droloxifene, toremifene, and

tamoxifen, as well as functional equivalents, analogs or derivatives thereof. These agents also inhibit or reduce TNF- $\alpha$  mediated NF $\kappa$ B activation. Moreover, as ER/NF $\kappa$ B modulators are not characterized by the undesirable side effect profile of GR/NF $\kappa$ B modulators at the doses used to treat autoimmune disorders, they are therefore amenable to chronic use in the prevention or treatment of autoimmune disorders.

When cells which have NF $\kappa$ B activity, such as human smooth muscle cells (SMCs), were cultured in the presence of 20% fetal calf serum (FCS) in Dulbecco's modification of Eagles' Medium (DMEM), more than 95% of NF $\kappa$ B was present in the cytoplasm, as determined by immunostaining for p65. When the cells were incubated with a pro-inflammatory cytokine (recombinant human TNF- $\alpha$  at 20 ng/ml) for 6 hours at 37° C., >80% of the NF $\kappa$ B was translocated to the nucleus. In cells which had been transfected with a reporter construct which has three consensus kB DNA elements fused to the luciferase gene, the amount of light which was produced when the cell lysate was exposed to luciferin and ATP was proportional to NF $\kappa$ B activity. Treatment of human SMCs for 6 hours with 20 ng/ml TGF- $\alpha$  resulted in a 19-fold increase in NF $\kappa$ B activity, consistent with the translocation of the NF $\kappa$ B complex to the nucleus.

In contrast, when human SMCs were treated with tamoxifen, NF $\kappa$ B remained in the cytoplasm and there was no detectable change in NF $\kappa$ B activity. When the human SMCs were incubated simultaneously with TNF- $\alpha$  (20 ng/ml) and tamoxifen (5  $\mu$ M), less than 10% of the NF $\kappa$ B was translocated to the nucleus and NF $\kappa$ B activity was stimulated by less than 3 fold. At this concentration, tamoxifen (TMX) inhibits TNF- $\alpha$ -induced NF $\kappa$ B activity by 86±12%.

Binding of tritium-labeled tamoxifen ( $^3$ H-TMX) was used to determine the affinity and number of binding sites for TMX in total cell lysates prepared from human VSMCs. Scatchard analysis of the binding of  $^3$ H-TMX revealed the presence of at least 3 distinct binding sites present at >1000 binding sites per cell. Site A had an affinity of 19 nM and was present at 4000 binding sites per cell. Site B had an affinity of 40 nM and was present at 9000 binding sites per cell and site C had an affinity of 3  $\mu$ M and was present at 100,000 binding sites per cell. These results are consistent with Site B being the human ER protein. It is likely that the higher affinity site A also contained the ER as it was efficiently immunoprecipitated by monoclonal antibodies directed against the human ER protein.

To identify other targets of TMX, an analog of 4-iodotamoxifen was covalently coupled to agarose and used to affinity purify antiestrogen binding proteins from total cell lysates prepared from human SMCs. Bound tamoxifen-binding proteins were eluted with the water-soluble quaternary tamoxifen salt N-methyl tamoxifen iodide. The eluting salt was removed by dialysis against Amberlite resin in phosphate buffer which irreversibly binds N-methyl tamoxifen. The affinity purified proteins were separated further by MonoQ ion exchange chromatography and fractions were assayed for  $^3$ H-TMX binding. Three peaks of protein associated with TMX-binding activity were identified. Peak I had an affinity of about 1  $\mu$ M and may correspond to site C. Further purification of this protein by gel filtration chromatography and gel electrophoresis allowed a molecular identification of the protein by N-terminal sequence analysis as human serum albumin. The amount of protein in the other two peaks of activity was less than the amount necessary to allow molecular characterization of these proteins.

Human SMC lysates were treated with a large excess of antibody directed against the human ER protein. After rotating overnight at 4° C., the antibody:antigen complex was precipitated by addition of 20  $\mu$ l/ml protein A/G agarose and centrifuged for 5 minutes at 4° C. The supernatant was treated similarly, until no further ER protein could be detected in the precipitated fraction. At concentrations of  $^3$ H-TMX below 50 nM (when the contribution by the low affinity site C should be negligible), all of the  $^3$ H-TMX binding sites were removed by treatment with the antibody directed against the human ER. Thus, both sites A and B (the high affinity sites) contain either the human ER or a protein which contains an epitope conserved with the human ER. It is very likely that both TMX binding complexes contain the ER.

To determine whether any ER protein in human VSMCs was complexed with other proteins, human SMCs cultured in DMEM+20% FCS were grown overnight in methionine-free DMEM+20% dialyzed FCS, then incubated with 50  $\mu$ Ci/ml  $^{35}$ S-labeled methionine in methionine-free DMEM+20% dialyzed FCS for 6 hours. Total cell lysates were prepared from the labeled cells and immunoprecipitated with antibodies to ER. The immunoprecipitated proteins were then analyzed by SDS gel electrophoresis and autoradiography. As expected, a band at 88 kDa corresponding to the ER protein was detected. Additionally, a band at 92 kDa was detected. Subsequent Western blotting determined that the 92 kDa band was the heat shock protein hsp90, which has been shown to be associated with ER in the cytoplasm. A third protein was also efficiently immunoprecipitated with the ER. This third protein migrated at 37–40 kDa. Since it has been shown that the GR steroid receptor interacts with NF $\kappa$ B transcription factor complexes, the 37–40 kDa protein was analyzed by Western blotting with antibodies directed against I $\kappa$ B-alpha. Human I $\kappa$ B-alpha has been reported to migrate as a 37 kDa protein. These experiments confirmed that human I $\kappa$ B-alpha forms complexes with human ER either as a ternary complex with hsp90 or with human ER alone.

Whole cell lysates from human SMCs were treated with antibody to I $\kappa$ B-alpha until no further I $\kappa$ B-alpha was found in the precipitated fraction. The supernatant had about 50% of the binding sites for  $^3$ H-TMX present in the original lysate, while immunoprecipitation with non-immune anti-serum did not reduce the number of TMX binding sites by more than 5%. Therefore, the three TMX binding sites in human SMC cell lysates are human serum albumin, a complex containing ER and I $\kappa$ B-alpha, and a complex containing ER but not I $\kappa$ B-alpha.

Because ER interacts with NF $\kappa$ B transcription factor complexes in a similar manner to that for GR, agents which modulate ER/NF $\kappa$ B interaction should modulate the inflammatory response without activating GR. To test this hypothesis, SMCs in DMEM+10% FCS were transfected with a vector comprising the MMTV LTR promoter coupled to the chloramphenicol acetyl transferase (CAT) gene and the neomycin resistance gene (neo). A stably transfected line was selected using geneticin. When these cells were treated with dexamethasone, expression of the CAT gene was elevated 3.7±0.7 fold. Treatment of these cells with concentrations of TNF- $\alpha$  (up to 100 ng/ml), tamoxifen (up to 10  $\mu$ M) or both agents together, did not stimulate expression of the CAT gene by more than 10%. Thus, ER/NF $\kappa$ B modulation would be expected to circumvent the undesirable side-effect profile associated with direct transcriptional activation by GR.

Tamoxifen may also upregulate expression of TGF-beta through its interaction with the NF $\kappa$ B transcription factor

complex, as suggested by the following observations. (1) The p68 RelB knockout mouse has a phenotype similar to the TGF-beta knockout mouse, suggesting that RelB may be important in the upregulation of TGF-beta that normally turns off acute inflammation, and (2) the kappa-like element in the rat TGF-beta-1 promoter is implicated in the tamoxifen-induced stimulation of TGF-beta expression. Thus, it is likely that a second consequence of ER/NFkB modulation by these agents is upregulation of TGF-beta expression. It is well known that TGF-beta has anti-inflammatory and immune-suppressive functions. Thus, the induction of TGF-beta by ER/NFkB modulating agents may act to synergistically reduce inflammation.

For ER/NFkB modulators, such as idoxifene, several exemplary dosing regimens are contemplated depending upon the particular autoimmune disease being treated and the stage to which the condition has progressed. For the treatment of incipient or early stage rheumatoid arthritis, when inflammation is evident but tissue damage is minimal, a low chronic oral dose of about 0.05 to about 10, preferably about 0.1 mg/kg/day, is employed. For local delivery, it is preferred that about 0.01 to about 1000 microgram per ml is administered, followed by chronic low dose oral delivery. When the disease progression is more severe, it is contemplated that a large loading dose, e.g., in the range of about 10 to about 100 mg/kg, is used to rapidly establish a therapeutic level of the ER/NFkB modulator in the circulation, followed by low chronic oral doses.

For the treatment of multiple sclerosis, an exemplary dose regimen is a single pre-loading dose, e.g., between about 10 to about 100 mg/kg, to establish a therapeutically effective amount of ER/NFkB modulator in the circulation, followed by a dose of about 0.1 to about 20, preferably about 0.5 to about 5, mg/kg/day.

ER/NFkB modulators that act to reduce or inhibit pathological inflammation associated with autoimmune disorders can be identified by the methods described hereinabove. Specifically, the agents may be identified by their ability to bind to NFkB/ER complexes, to inhibit NFkB activation induced by TNF-alpha and/or other pro-inflammatory cytokines, and to prevent activation of autoreactive T lymphocytes.

#### EXAMPLE VIII

##### Effects of the Therapeutic Agents on Cholesterol Levels

Twenty six patients with high cholesterol were administered simvastatin for 16 weeks. Blood was collected at six time points during the 16 weeks and analyzed for TGF-beta levels. While serum cholesterol levels were reduced in these patients, there was no effect on TGF-beta levels in any of the patients. In contrast, some of the patients participating in a trial in which tamoxifen, a tamoxifen analog, or placebo, was administered, showed significant decreases in cholesterol levels. Therefore, a combination of one of the therapeutic agents of the invention and an agent which lowers serum cholesterol levels may exert a synergistic effect and thus, may be useful in the practice in the methods of the invention. Moreover, therapeutic agents of the invention alone may be useful to lower serum cholesterol levels.

#### EXAMPLE IX

##### Assay for Measuring Free Anti-sR11 antibody in Human Serum

Recombinant sR11 was coated onto the bottom of high protein binding ELISA plates for two hours in 50 mM

carbonate buffer (pH 9), then washed, and non-specific binding blocked using 5% Tween-20 in 5% sucrose in water, containing 0.02% sodium azide (TSA block). Various serum and plasma samples were then incubated with the coated wells for 2 hours at room temperature with shaking. Unbound serum components were washed off using TBS plus 0.05% Tween-20 with four washing cycles ensuring complete aspiration of the well between each cycle. Any bound human immunoglobulin was then detected by adding anti-human-IgG antibodies coupled to horseradish peroxidase in wash buffer for one hour. Bound peroxidase was visualized using TMB substrate. All normal sera tested contained detectable levels of IgG antibodies binding to sR11. This signal was eliminated by omitting any one of: sR11 antigen, serum or anti-human-IgG peroxidase. This confirms the presence of high affinity autoantibodies directed against the extracellular domain of the human type II TGF-beta receptor.

It was also determined that human sera from normal healthy individuals does not contain autoantibodies against a wide variety of other human proteins, including, but not limited to, fibrinogen, factor II, complement C4, spilloprotein (a), collagen type I, III and IV or the extracellular domain of the human IL-10 receptor (a receptor expressed on endothelial cells). This data suggests that in normal humans there is a relatively specific autoimmune response to the TGF-beta type II receptor extracellular domain.

Antibodies of the IgG class against sR11 are also present in normal human serum. Although there may be antibodies of the IgM class, interference from rheumatoid factor (IgM directed against IgG) cannot be excluded at this time. Analysis of the anti-sR11 IgG using IgG sub-class specific detection antisera has demonstrated that the majority of IgG reacting with sR11 in normal human serum is of the IgG2 sub-class. Thus, to measure antibodies against sR11, ELISA plate wells are coated with recombinant or purified human sR11 or an immunogenic portion thereof. Wells are blocked against non-specific binding using a blocking agent for the particular sample type, e.g. for serum analysis, a TCA block. Serum is added to the well, preferably undiluted and untreated. Plasma or other bodily fluids may also be used. The wells are washed to remove unbound components and the bound anti-sR11 Ig is detected using an appropriate anti-human Ig antiserum, labeled for detection.

Because the assay does not determine absolute levels of antibody, the signal is referenced to a large pool of normal serum (PNS). A standard curve is constructed for each assay using sequential dilutions of PNS. PNS is arbitrarily designated to have 100 units of anti-sR11 Ig, e.g., IgG.

Results:	
Healthy normals	Median 100 units 95% of individuals in the range 50 to 200 units
NCA's	Median 120 units 95% of individuals in the range 50 to 250 units
TVDs	Median 15 units 95% of individuals in the range <10 to 50 units

A result of <50 units on the detection of human pan IgG indicates the presence of atherosclerosis, with high sensitivity (>95%) and probably similar specificity. However, it is envisioned that the detection of other classes or subclasses, IgG2, may be useful to detect diseases characterized by endothelial cell activation, or a specific disease.

Body fluids that contain detectable levels of immunoglobulin may be used, e.g., plasma or serum. Samples can be

fresh or frozen. The anti-sR11 Ig is stable over time for a given individual (intraperson variation on a 3-month time scale is <10% of the interperson variation). Accurate diagnosis can therefore be achieved on single sample from a given individual. Moreover, the Ig are stable to multiple cycles of freeze thawing and to long storage times at -20° C. However, the assay is still subject to capture interference by subclass or classes of immunoglobulin not otherwise detected. For example, when using anti-human-IgG peroxidase as the detection antiserum, the assay may detect little or no IgG against sR11 because of the presence of large amounts of IgG against sR11 occupying all the available antigen sites.

The levels of anti-sR11-IgG in serum and plasma samples derived from individuals with severe coronary atherosclerosis, defined by coronary angiography and individuals with normal coronary angiograms, were measured. Patients with atherosclerosis (TVD patients) had approximately a five fold lower median concentration of anti-sR11-IgG compared with individuals with normal coronary arteries (NCA individuals). The absolute amount of sR11 IgG could not readily be determined, but the relative amounts compared with pooled normal serum could be determined by running various dilutions of pooled normal serum as a standard curve with each assay. In all cases a standard pooled serum was used and this serum was arbitrarily designated to have 100 units of anti-sR11 IgG.

Based on this standardization, the median concentration of anti sR11 IgG among 100 individuals with coronary atherosclerosis was 14.6 units, compared with 84.9 units among the individuals with normal coronary arteries. This difference was highly statistically significant ( $p < 0.001$ ; Mann-Whitney U-test). The detection limit for the ELISA as performed under these conditions was approximately 10 units of anti-sR11-IgG. As a consequence, fully 40% of the patients with atherosclerosis had levels at or below the detection limit of the assay, whereas all of the individuals with normal coronary arteries had detectable levels. The sensitivity and specificity of this test are estimated to be greater than 90%. As a result, measurement of anti-sR11 IgG using this assay has far greater diagnostic potential than any existing plasma or serum biochemical marker for coronary heart disease.

This method can conveniently be used to diagnosis the presence of the disease (e.g. atherosclerosis), determine the extent of disease, evaluate prognosis (i.e. determine future risk prior to onset of symptoms), or to monitor the effectiveness of a treatment.

The suppressed levels of anti-sR11 IgG in plasma and serum from individuals with atherosclerosis may be due to (a) lower levels of anti-sR11 IgG, which assumes that lower detection of anti-sR11 IgG results from the presence of lower levels of the IgG; (b) increased levels of anti-sR11 IgG, or other non IgG classes, as the assays are subject to inhibition by non-IgG class anti-sR11 antibodies; or (c) increased levels of sR11 antigen. The sR11 antigen which is normally expressed on endothelial cells may be shed during phenotypic changes in endothelial cell gene expression pattern, e.g., during activation, a process thought to occur in atherogenesis. sR11 in plasma would then form complexes with the anti-sR11 antibodies and make them more difficult to detect. As a result, lower levels of anti-sR11 IgG would be detectable in individuals with increased endothelial cell activation.

MacCaffrey and colleagues have reported a switch from expression of TGF- $\beta$  type II receptor to TGF- $\beta$  type I receptor during the development of atherosclerosis in man,

and one mechanism which might contribute to this switch would be shedding of sR11 from endothelial cells as they become activated (MacCaffrey et al. *J. Clin. Invest.* 1996, 2667-2675). Thus, plasma concentrations of sR11 may be a direct measure of the state of endothelial cell activation (related, for example, to functional tests of endothelial cells function, e.g., brachial reactivity). Since the presence of sR11 is expected to reduce detection of any anti-sR11-IgG present in the plasma (by forming complexes with it), the level of anti-sR11-IgG would be a proxy measure for levels of sR11 antigen (i.e., low levels of anti-sR11-IgG result from high levels of sR11 antigen, resulting, in turn, from endothelial cell activation). Thus, this assay represents the first useful plasma measure of endothelial cell function, and thus, is a measure of an individual at risk of or having a disease characterized by endothelial cell activation. Moreover, the assay offers many advantages over the low throughput endothelial cell function assays such as brachial reactivity currently being used.

In addition to the assay described above, the methodology described herein can also be utilized to carry out the following assays:

(a) Detection of free sR11 antigen	High levels diagnostic for very severe atherosclerosis
(b) Detection of sR11:anti-sR11 complexes	High level diagnostic of moderate to very severe atherosclerosis
(c) Detection of total sR11 antigen	Diagnostic of extent of endothelial cell activation and hence of atherosclerotic disease progression.

The methodology described herein can also be used to determine the level (e.g. the relative presence or absence) of TGF- $\beta$  type II receptors (e.g. the extracellular domain of the TGF- $\beta$  type II receptor) in mammalian cells or tissue. Endothelial cells are believed to shed the extracellular domain of the TGF- $\beta$  type II receptor during activation, and there is believed to be a correlation between endothelial cell activation and atherogenesis, as well as other diseases. Accordingly, the invention also provides a method comprising detecting TGF- $\beta$  type II receptors in mammalian cells or tissue, by combining the cells or tissue with a capture moiety that binds TGF- $\beta$  type II receptors or a portion thereof, forming a capture complex, and detecting or determining the amount of the capture complex.

All publications and patents are incorporated by reference herein, as though individually incorporated by reference, as long as they are not inconsistent with the present disclosure. The invention is not limited to the exact details shown and described, for it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention defined by the claims.

What is claimed is:

1. A therapeutic method for preventing or treating a condition or symptom associated with Marfan's syndrome, fibrosis, or senile dementia, comprising administering to a mammal in need of such therapy, an effective amount of a compound of formula VI:



wherein

$R^6$  is  $(C_1-C_6)$ alkyl, or aryl, optionally substituted by 1, 2, or 3  $V_i$ ;

$R^7$  is phenyl, optionally substituted by 1, 2, or 3  $V_i$ ; or  $R^7$  is  $(C_1-C_{12})$ alkyl, halo $(C_1-C_{12})$ alkyl,  $(C_3-C_6)$ cycloalkyl,  $(C_1-C_6)$ alkylcyclo $(C_1-C_6)$ alkyl,  $(C_3-C_6)$ cycloalkenyl, or  $(C_1-C_6)$ alkyl $(C_3-C_6)$ cycloalkenyl;

$R^8$  is hydrogen or phenyl, optionally substituted by 1, 2, or 3  $V_i$ ;

$R^9$  is hydrogen, halo, aryl, aryl $(C_1-C_6)$ alkyl, halo $(C_1-C_{12})$ alkyl, cyano $(C_1-C_{12})$ alkyl, or  $(C_1-C_{12})$ alkyl, wherein any aryl may optionally be substituted by 1, 2, or 3  $V_i$ ; or

— is a single bond or is  $-C(B)(D)-$ , wherein B and D are each independently hydrogen,  $(C_1-C_6)$ alkyl, or halo;

V is  $OPO_3H_2$ ,  $(C_1-C_6)$ alkyl,  $(C_1-C_6)$ alkoxy, mercapto,  $(C_1-C_6)$ alkylthio, halo, trifluoromethyl, pentafluoroethyl, nitro,  $N(R_1)(R_2)$ , cyano, trifluoromethoxy, pentafluoromethoxy, benzoyl, hydroxy,  $-(CH_2)_{0-4}C(=O)(C_1-C_6)$ alkyl,  $-UC(=O)(C_1-C_6)$ alkyl, benzyl,  $-OSO_2(CH_2)_0-4CH_3$ ,  $-U(CH_2)_2COOR_1$ ,  $-(CH_2)_0-4COOR_1$ ,  $-U(CH_2)_2OR_1$ ,  $-(CH_2)_0-4OR_1$ ,  $-U(CH_2)_2C(=O)R_1$ ,  $-(CH_2)_0-4C(=O)R_1$ ,  $-(CH_2)_0-4OC(=O)R_1$ , wherein U is O,  $N(R_m)$ , or S;

Z is  $-(CH_2)_{1-3}$ ,  $-O-$ ,  $-OCH_2-$ ,  $-CH_2O-$ ,  $-C(=O)O-$ ,  $-N(R_3)-$ ,  $C=O$ , or a covalent bond;

$R_1$  is amino, optionally substituted with one or two  $(C_1-C_6)$ alkyl; or an N-heterocyclic ring optionally containing 1 or 2 additional  $N(R_1)$ , S, or nonperoxide O, wherein  $R_1$  is H  $(C_1-C_6)$ alkyl, phenyl, or benzyl;

$R_m$  and  $R_n$  are independently hydrogen,  $(C_1-C_6)$ alkyl, phenyl, benzyl, or  $(C_1-C_6)$ alkanoyl; or  $R_m$  and  $R_n$  together with the nitrogen to which they are attached are a 3, 4, 5, or 6 membered heterocyclic ring;

$R_p$  is H or  $(C_1-C_6)$ alkyl; and

$R_m$  and  $R_n$  are independently hydrogen,  $(C_1-C_6)$ alkyl, phenyl, benzyl, or  $(C_1-C_6)$ alkanoyl; the compound is MER25;

or a pharmaceutically acceptable salt thereof.

2. A therapeutic method for preventing or treating a condition or symptom associated with Parkinson's disease comprising administering to a mammal in need of such therapy, an effective amount of a compound of formula VI:



wherein

$R^6$  is  $(C_1-C_6)$ alkyl, or aryl, optionally substituted by 1, 2, or 3  $V_i$ ;

$R^7$  is phenyl, optionally substituted by 1, 2, or 3  $V_i$ ; or  $R^7$  is  $(C_1-C_{12})$ alkyl, halo $(C_1-C_{12})$ alkyl,  $(C_3-C_6)$ cycloalkyl,  $(C_1-C_6)$ alkylcyclo $(C_1-C_6)$ alkyl,  $(C_3-C_6)$ cycloalkenyl, or  $(C_1-C_6)$ alkyl $(C_3-C_6)$ cycloalkenyl;

$R^8$  is hydrogen or phenyl, optionally substituted by 1, 2, or 3  $V_i$ ;

$R^9$  is hydrogen, halo, aryl, aryl $(C_1-C_6)$ alkyl, halo $(C_1-C_{12})$ alkyl, cyano $(C_1-C_{12})$ alkyl, or  $(C_1-C_{12})$ alkyl, wherein any aryl may optionally be substituted by 1, 2, or 3  $V_i$ ; or

— is a single bond or is  $-C(B)(D)-$ , wherein B and D are each independently hydrogen,  $(C_1-C_6)$ alkyl, or halo;

V is  $OPO_3H_2$ ,  $(C_1-C_6)$ alkyl,  $(C_1-C_6)$ alkoxy, mercapto,  $(C_1-C_6)$ alkylthio, halo, trifluoromethyl, pentafluoroethyl, nitro,  $N(R_1)(R_2)$ , cyano, trifluoromethoxy, pentafluoromethoxy, benzoyl, hydroxy,  $-(CH_2)_{0-4}C(=O)(C_1-C_6)$ alkyl,  $-UC(=O)(C_1-C_6)$ alkyl, benzyl,  $-OSO_2(CH_2)_0-4CH_3$ ,  $-U(CH_2)_2COOR_1$ ,  $-(CH_2)_0-4COOR_1$ ,  $-U(CH_2)_2OR_1$ ,  $-(CH_2)_0-4OR_1$ ,  $-U(CH_2)_2C(=O)R_1$ ,  $-(CH_2)_0-4C(=O)R_1$ ,  $-(CH_2)_0-4OC(=O)R_1$ , wherein U is O,  $N(R_m)$ , or S;

Z is  $-(CH_2)_{1-3}$ ,  $-O-$ ,  $-OCH_2-$ ,  $-CH_2O-$ ,  $-C(=O)O-$ ,  $-N(R_3)-$ ,  $C=O$ , or a covalent bond;

$R_1$  is amino, optionally substituted with one or two  $(C_1-C_6)$ alkyl; or an N-heterocyclic ring optionally containing 1 or 2 additional  $N(R_1)$ , S, or nonperoxide O, wherein  $R_1$  is H  $(C_1-C_6)$ alkyl, phenyl, or benzyl;

$R_m$  and  $R_n$  are independently hydrogen,  $(C_1-C_6)$ alkyl, phenyl, benzyl, or  $(C_1-C_6)$ alkanoyl; or  $R_m$  and  $R_n$  together with the nitrogen to which they are attached are a 3, 4, 5, or 6 membered heterocyclic ring;

$R_p$  is H or  $(C_1-C_6)$ alkyl; and

$R_m$  and  $R_n$  are independently hydrogen,  $(C_1-C_6)$ alkyl, phenyl, benzyl, or  $(C_1-C_6)$ alkanoyl;

the compound is MER25;

or a pharmaceutically acceptable salt thereof.

3. A therapeutic method for preventing or treating a condition or symptom associated with Alzheimer's disease comprising administering to a mammal in need of such therapy, an effective amount of a compound of formula VI:



(vi)

wherein

$R^6$  is  $(C_1-C_6)$ alkyl, or aryl, optionally substituted by 1, 2, or 3  $V_i$ ;

$R^7$  is phenyl, optionally substituted by 1, 2, or 3  $V_i$ ; or  $R^7$  is  $(C_1-C_{12})$ alkyl, halo $(C_1-C_{12})$ alkyl,  $(C_3-C_6)$ cycloalkyl,  $(C_1-C_6)$ alkylcyclo $(C_1-C_6)$ alkyl,  $(C_3-C_6)$ cycloalkenyl, or  $(C_1-C_6)$ alkyl $(C_3-C_6)$ cycloalkenyl;

$R^8$  is hydrogen or phenyl, optionally substituted by 1, 2, or 3  $V_i$ ;

$R^9$  is hydrogen, halo, aryl, aryl $(C_1-C_6)$ alkyl, halo $(C_1-C_{12})$ alkyl, cyano $(C_1-C_{12})$ alkyl, or  $(C_1-C_{12})$ alkyl, wherein any aryl may optionally be substituted by 1, 2, or 3  $V_i$ ; or

— is a single bond or is —C(B)(D)—, wherein B and D are each independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, or halo;

V is OPO<sub>3</sub>H<sub>2</sub>, (C<sub>1</sub>-C<sub>6</sub>)alkyl, (C<sub>1</sub>-C<sub>6</sub>)alkoxy, mercapto, (C<sub>1</sub>-C<sub>6</sub>)alkylthio, halo, trifluoromethyl, pentafluoroethyl, nitro, N(R<sub>4</sub>)(R<sub>5</sub>), cyano, trifluoromethoxy, pentafluoroethoxy, benzoyl, hydroxy, (CH<sub>2</sub>)<sub>0-4</sub>C(=O)(C<sub>1</sub>-C<sub>6</sub>)alkyl, —UC(=O)(C<sub>1</sub>-C<sub>6</sub>)alkyl, benzyl, —OSO<sub>2</sub>(CH<sub>2</sub>)<sub>0-4</sub>CH<sub>3</sub>, —U(CH<sub>2</sub>)<sub>1-4</sub>COOR<sub>6</sub>, —(CH<sub>2</sub>)<sub>0-4</sub>COOR<sub>6</sub>, —U(CH<sub>2</sub>)<sub>1-4</sub>OR<sub>6</sub>, —(CH<sub>2</sub>)<sub>0-4</sub>OR<sub>6</sub>, —U(CH<sub>2</sub>)<sub>1-4</sub>C(=O)R<sub>6</sub>, —(CH<sub>2</sub>)<sub>0-4</sub>C(=O)R<sub>6</sub>, —U(CH<sub>2</sub>)<sub>1-4</sub>R<sub>6</sub>, —(CH<sub>2</sub>)<sub>0-4</sub>R<sub>6</sub>, or —U(CH<sub>2</sub>)<sub>0-4</sub>OC(=O)R<sub>6</sub>; wherein U is O, N(R<sub>4</sub>), or S;

Z is —(CH<sub>2</sub>)<sub>1-3</sub>—, —O—, —OCH<sub>2</sub>—, —CH<sub>2</sub>O—, —C(=O)O—, —N(R<sub>4</sub>)—, C=O, or a covalent bond;

R<sub>4</sub> is amino, optionally substituted with one or two (C<sub>1</sub>-C<sub>6</sub>)alkyl; or an N-heterocyclic ring optionally containing 1 or 2 additional N(R<sub>4</sub>), S, or nonperoxide O, wherein R<sub>4</sub> is H or (C<sub>1</sub>-C<sub>6</sub>)alkyl, phenyl, or benzyl;

R<sub>5</sub> and R<sub>6</sub> are independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, phenyl, benzyl, or (C<sub>1</sub>-C<sub>6</sub>)alkanoyl; or R<sub>5</sub> and R<sub>6</sub> together with the nitrogen to which they are attached are a 3, 4, 5, or 6 membered heterocyclic ring;

R<sub>6</sub> is H or (C<sub>1</sub>-C<sub>6</sub>)alkyl; and

R<sub>4</sub> and R<sub>6</sub> are independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, phenyl, benzyl, or (C<sub>1</sub>-C<sub>6</sub>)alkanoyl;

the compound is MER25;

or a pharmaceutically acceptable salt thereof;

provided the compound of formula VI is not toremifene, tamoxifen, monophenoltamoxifen, 4-hydroxytoremifene, clomifene, 4-hydroxytamoxifen, 3-hydroxytamoxifen, N-desmethyltamoxifen, cyanotamoxifen, N-desmethyltoremifene, monophenoltoremifene, or desaminotoremifene.

4. A therapeutic method for preventing or treating a condition or symptom associated with an autoimmune disease comprising administering to a mammal in need of such therapy, an effective amount of a compound of formula VI:



wherein

R<sup>4</sup> is (C<sub>1</sub>-C<sub>6</sub>)alkyl, or aryl, optionally substituted by 1, 2, or 3 V; or

R<sup>7</sup> is phenyl, optionally substituted by 1, 2, or 3 V; or R<sup>4</sup> is (C<sub>1</sub>-C<sub>12</sub>)alkyl, halo(C<sub>1</sub>-C<sub>12</sub>)alkyl, (C<sub>3</sub>-C<sub>6</sub>)cycloalkyl, (C<sub>1</sub>-C<sub>6</sub>)alkylcyclo(C<sub>1</sub>-C<sub>6</sub>)alkyl, (C<sub>3</sub>-C<sub>6</sub>)cycloalkenyl, or (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>3</sub>-C<sub>6</sub>)cycloalkenyl;

R<sup>5</sup> is hydrogen or phenyl, optionally substituted by 1, 2, or 3 V;

R<sup>6</sup> is hydrogen, halo, aryl, aryl(C<sub>1</sub>-C<sub>6</sub>)alkyl, halo(C<sub>1</sub>-C<sub>12</sub>)alkyl, cyano(C<sub>1</sub>-C<sub>12</sub>)alkyl, or (C<sub>1</sub>-C<sub>12</sub>)alkyl, wherein any aryl may optionally be substituted by 1, 2, or 3 V; or

— is a single bond or is —C(B)(D)—, wherein B and D are each independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, or halo;

V is OPO<sub>3</sub>H<sub>2</sub>, (C<sub>1</sub>-C<sub>6</sub>)alkyl, (C<sub>1</sub>-C<sub>6</sub>)alkoxy, mercapto, (C<sub>1</sub>-C<sub>6</sub>)alkylthio, halo, trifluoromethyl,

pentafluoroethyl, nitro, N(R<sub>4</sub>)(R<sub>5</sub>), cyano, trifluoromethoxy, pentafluoroethoxy, benzoyl, hydroxy, —(CH<sub>2</sub>)<sub>0-4</sub>C(=O)(C<sub>1</sub>-C<sub>6</sub>)alkyl, —UC(=O)(C<sub>1</sub>-C<sub>6</sub>)alkyl, benzyl, —OSO<sub>2</sub>(CH<sub>2</sub>)<sub>0-4</sub>CH<sub>3</sub>, —U(CH<sub>2</sub>)<sub>1-4</sub>COOR<sub>6</sub>, —(CH<sub>2</sub>)<sub>0-4</sub>COOR<sub>6</sub>, —U(CH<sub>2</sub>)<sub>1-4</sub>OR<sub>6</sub>, —(CH<sub>2</sub>)<sub>0-4</sub>OR<sub>6</sub>, —U(CH<sub>2</sub>)<sub>1-4</sub>C(=O)R<sub>6</sub>, —(CH<sub>2</sub>)<sub>0-4</sub>C(=O)R<sub>6</sub>, —U(CH<sub>2</sub>)<sub>1-4</sub>R<sub>6</sub>, —(CH<sub>2</sub>)<sub>0-4</sub>R<sub>6</sub>, or —U(CH<sub>2</sub>)<sub>0-4</sub>OC(=O)R<sub>6</sub>; wherein U is O, N(R<sub>4</sub>), or S;

Z is —(CH<sub>2</sub>)<sub>1-3</sub>—, —O—, —OCH<sub>2</sub>—, —CH<sub>2</sub>O—, —C(=O)O—, —N(R<sub>4</sub>)—, C=O, or a covalent bond;

R<sub>4</sub> is amino, optionally substituted with one or two (C<sub>1</sub>-C<sub>6</sub>)alkyl; or an N-heterocyclic ring optionally containing 1 or 2 additional N(R<sub>4</sub>), S, or nonperoxide O, wherein R<sub>4</sub> is H or (C<sub>1</sub>-C<sub>6</sub>)alkyl, phenyl, or benzyl;

R<sub>5</sub> and R<sub>6</sub> are independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, phenyl, benzyl, or (C<sub>1</sub>-C<sub>6</sub>)alkanoyl; or R<sub>5</sub> and R<sub>6</sub> together with the nitrogen to which they are attached are a 3, 4, 5, or 6 membered heterocyclic ring;

R<sub>6</sub> is H or (C<sub>1</sub>-C<sub>6</sub>)alkyl; and

R<sub>5</sub> and R<sub>6</sub> are independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, phenyl, benzyl, or (C<sub>1</sub>-C<sub>6</sub>)alkanoyl;

the compound is MER25;

or a pharmaceutically acceptable salt thereof;

provided that the compound is not toremifene, tamoxifen, 4-hydroxytamoxifen, 3-hydroxytamoxifen, 4-hydroxytoremifene or N-desmethyltoremifene.

5. The method of claim 4 wherein the autoimmune disease is lupus erythematosus.

6. The method of claim 4 wherein the autoimmune disease is rheumatoid arthritis.

7. The method of claim 4 wherein the autoimmune disease is multiple sclerosis.

8. A therapeutic method for lowering serum cholesterol comprising administering to a mammal in need of such therapy, an effective amount of a compound of formula VI:



wherein

R<sup>4</sup> is (C<sub>1</sub>-C<sub>6</sub>)alkyl, or aryl, optionally substituted by 1, 2, or 3 V;

R<sup>7</sup> is phenyl, optionally substituted by 1, 2, or 3 V; or R<sup>4</sup> is (C<sub>1</sub>-C<sub>12</sub>)alkyl, halo(C<sub>1</sub>-C<sub>12</sub>)alkyl, (C<sub>3</sub>-C<sub>6</sub>)cycloalkyl, (C<sub>1</sub>-C<sub>6</sub>)alkylcyclo(C<sub>1</sub>-C<sub>6</sub>)alkyl, (C<sub>3</sub>-C<sub>6</sub>)cycloalkenyl, or (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>3</sub>-C<sub>6</sub>)cycloalkenyl;

R<sup>5</sup> is hydrogen or phenyl, optionally substituted by 1, 2, or 3 V;

R<sup>6</sup> is hydrogen, halo, aryl, aryl(C<sub>1</sub>-C<sub>6</sub>)alkyl, halo(C<sub>1</sub>-C<sub>12</sub>)alkyl, cyano(C<sub>1</sub>-C<sub>12</sub>)alkyl, or (C<sub>1</sub>-C<sub>12</sub>)alkyl, wherein any aryl may optionally be substituted by 1, 2, or 3 V; or

— is a single bond or is —C(B)(D)—, wherein B and D are each independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, or halo;

V is OPO<sub>3</sub>H<sub>2</sub>, (C<sub>1</sub>-C<sub>6</sub>)alkyl, (C<sub>1</sub>-C<sub>6</sub>)alkoxy, mercapto, (C<sub>1</sub>-C<sub>6</sub>)alkylthio, halo, trifluoromethyl, pentafluoroethyl, nitro, N(R<sub>4</sub>)(R<sub>5</sub>), cyano, trifluoromethoxy, pentafluoroethoxy, benzoyl, hydroxy, —(CH<sub>2</sub>)<sub>0-4</sub>C(=O)(C<sub>1</sub>-C<sub>6</sub>)alkyl, —UC(=O)(C<sub>1</sub>-C<sub>6</sub>)alkyl, benzyl, —OSO<sub>2</sub>(CH<sub>2</sub>)<sub>0-4</sub>CH<sub>3</sub>, —U(CH<sub>2</sub>)<sub>1-4</sub>COOR<sub>6</sub>, —(CH<sub>2</sub>)<sub>0-4</sub>COOR<sub>6</sub>, —U(CH<sub>2</sub>)<sub>1-4</sub>OR<sub>6</sub>, —(CH<sub>2</sub>)<sub>0-4</sub>OR<sub>6</sub>, —U(CH<sub>2</sub>)<sub>1-4</sub>C(=O)R<sub>6</sub>, —(CH<sub>2</sub>)<sub>0-4</sub>C(=O)R<sub>6</sub>, —U(CH<sub>2</sub>)<sub>1-4</sub>R<sub>6</sub>, —(CH<sub>2</sub>)<sub>0-4</sub>R<sub>6</sub>, or —U(CH<sub>2</sub>)<sub>0-4</sub>OC(=O)R<sub>6</sub>; wherein U is O, N(R<sub>4</sub>), or S;

4.  $\text{COOR}_p$ ,  $-(\text{CH}_2)_n\text{COOR}_p$ ,  $-\text{U}(\text{CH}_2)_{2-4}\text{OR}_p$ ,  
 $-(\text{CH}_2)_6\text{OR}_p$ ,  $-\text{U}(\text{CH}_2)_4\text{C}(=\text{O})\text{R}_q$ ,  $-(\text{CH}_2)_6\text{C}$   
 $(=\text{O})\text{R}_q$ ,  $-\text{U}(\text{CH}_2)_4\text{R}_q$ ,  $-(\text{CH}_2)_6\text{R}_q$ , or  
 $-\text{U}(\text{CH}_2)_2\text{OC}(=\text{O})\text{R}_p$  wherein U is O,  $\text{N}(\text{R}_m)_2$ , or S;  
 Z is  $-(\text{CH}_2)_{3-5}$ ,  $-\text{O}-$ ,  $-\text{OCH}_2-$ ,  $-\text{CH}_2\text{O}-$ ,  
 $-\text{C}(=\text{O})\text{O}-$ ,  $-\text{N}(\text{R}_q)-$ ,  $\text{C}=\text{O}$ , or a covalent bond;  
 $\text{R}_3$  is amino, optionally substituted with one or two  
 $(\text{C}_1-\text{C}_6)\text{alkyl}$  or an N-heterocyclic ring optionally con-  
 taining 1 or 2 additional  $\text{N}(\text{R}_q)$ , S, or nonperoxide O,  
 wherein  $\text{R}_3$  is  $\text{H}(\text{C}_1-\text{C}_6)\text{alkyl}$ , phenyl, or benzyl;  
 $\text{R}_m$  and  $\text{R}_q$  are independently hydrogen,  $(\text{C}_1-\text{C}_6)\text{alkyl}$ ,  
 phenyl, benzyl, or  $(\text{C}_1-\text{C}_6)\text{alkenyl}$ ; or  $\text{R}_m$  and  $\text{R}_q$   
 together with the nitrogen to which they are attached  
 are a 3, 4, 5, or 6 membered heterocyclic ring;  
 $\text{R}_p$  is H or  $(\text{C}_1-\text{C}_6)\text{alkyl}$ ; and  
 $\text{R}_m$  and  $\text{R}_q$  are independently hydrogen,  $(\text{C}_1-\text{C}_6)\text{alkyl}$ ,  
 phenyl, benzyl, or  $(\text{C}_1-\text{C}_6)\text{alkenyl}$ ;  
 the compound is MER25;  
 or a pharmaceutically acceptable salt thereof;  
 provided the compound of formula VI is not tamoxifen;  
 and provided  $\text{R}^7$  is not halo when Z is a covalent bond;  $\text{R}^6$   
 is a phenyl radical or a phenyl radical substituted only  
 at the 4-position with a group  $-\text{O}(\text{CH}_2)_2\text{R}^7$ ;  $\text{R}^7$  is a  
 phenyl radical or a phenyl radical substituted with one  
 halogen or  $(\text{C}_1-\text{C}_6)\text{alkyl}$ ; and  $\text{R}^8$  is a phenyl radical or  
 a phenyl radical substituted only at the 4-position with  
 a group  $-\text{O}(\text{CH}_2)_2\text{R}^6$ ; and — is a single bond;  
 and provided that  $\text{R}^6$  is not ethyl when Z is a covalent  
 bond;  $\text{R}^6$  is a phenyl radical substituted only at the  
 4-position with a group  $-\text{O}(\text{CH}_2)_2\text{R}^8$ ;  $\text{R}_8$  is  
 methylamino, ethylamino, dimethylamino or diethyl-  
 amino;  $\text{R}^7$  is unsubstituted phenyl; and  $\text{R}^8$  is  
 3-hydroxyphenyl; and — is a single bond.  
 9. A therapeutic method for enhancing or promoting  
 wound healing, comprising administering to a mammal in  
 need of such therapy, an effective amount of a compound of  
 formula VI:

(VI)



wherein

$\text{R}^6$  is  $(\text{C}_1-\text{C}_6)\text{alkyl}$ , or aryl, optionally substituted by 1, 2,  
 or 3  $\text{V}$ ;  
 $\text{R}^7$  is phenyl, optionally substituted by 1, 2, or 3  $\text{V}$ ; or  $\text{R}^7$   
 is  $(\text{C}_1-\text{C}_{12})\text{alkyl}$ , halo  $(\text{C}_1-\text{C}_{12})\text{alkyl}$ ,  $(\text{C}_3-\text{C}_6)$   
 cycloalkyl,  $(\text{C}_1-\text{C}_6)\text{alkylcyclo}(\text{C}_1-\text{C}_6)\text{alkyl}$ ,  $(\text{C}_3-\text{C}_6)$   
 cycloalkenyl, or  $(\text{C}_1-\text{C}_6)\text{alkyl}(\text{C}_3-\text{C}_6)\text{cycloalkenyl}$ ;  
 $\text{R}^8$  is hydrogen or phenyl, optionally substituted by 1, 2,  
 or 3  $\text{V}$ ;  
 $\text{R}^9$  is hydrogen, halo, aryl, aryl  $(\text{C}_1-\text{C}_6)\text{alkyl}$ , halo  
 $(\text{C}_1-\text{C}_{12})\text{alkyl}$ , cyano  $(\text{C}_1-\text{C}_{12})\text{alkyl}$ , or  $(\text{C}_1-\text{C}_{12})\text{alkyl}$ ,  
 wherein any aryl may optionally be substituted by 1, 2,  
 or 3  $\text{V}$ ; or  
 — is a single bond or is  $-\text{C}(\text{B})(\text{D})-$ , wherein B and D  
 are each independently hydrogen,  $(\text{C}_1-\text{C}_6)\text{alkyl}$ , or  
 halo;  
 $\text{V}$  is  $\text{OPO}(\text{H})_2$ ,  $(\text{C}_1-\text{C}_6)\text{alkyl}$ ,  $(\text{C}_1-\text{C}_6)\text{alkoxy}$ , mercapto,  
 $(\text{C}_1-\text{C}_6)\text{alkylthio}$ , halo, trifluoromethyl,  
 pentafluoroethyl, nitro,  $\text{N}(\text{R}_m)(\text{R}_q)$ , cyano,

trifluoromethoxy, pentafluoroethoxy, benzoyl, hydroxy,  
 $-(\text{CH}_2)_6\text{C}(=\text{O})(\text{C}_1-\text{C}_6)\text{alkyl}$ ,  $-\text{U}(\text{C}(=\text{O})(\text{C}_1-\text{C}_6)$   
 $\text{alkyl})$ , benzyl,  $-\text{OSO}_2(\text{CH}_2)_6\text{CH}_3$ ,  $-\text{U}(\text{CH}_2)_{2-4}$   
 $\text{COOR}_p$ ,  $-(\text{CH}_2)_6\text{COOR}_p$ ,  $-\text{U}(\text{CH}_2)_2\text{OR}_p$ ,  
 $-(\text{CH}_2)_6\text{OR}_p$ ,  $-\text{U}(\text{CH}_2)_4\text{C}(=\text{O})\text{R}_q$ ,  $-(\text{CH}_2)_6\text{C}$   
 $(=\text{O})\text{R}_q$ ,  $-\text{U}(\text{CH}_2)_4\text{R}_q$ ,  $-(\text{CH}_2)_6\text{R}_q$ , or  
 $-\text{U}(\text{CH}_2)_2\text{OC}(=\text{O})\text{R}_p$  wherein U is O,  $\text{N}(\text{R}_m)_2$ , or S;  
 Z is  $-(\text{CH}_2)_{3-5}$ ,  $-\text{O}-$ ,  $-\text{OCH}_2-$ ,  $-\text{CH}_2\text{O}-$ ,  
 $-\text{C}(=\text{O})\text{O}-$ ,  $-\text{N}(\text{R}_q)-$ ,  $\text{C}=\text{O}$ , or a covalent bond;  
 $\text{R}_3$  is amino, optionally substituted with one or two  
 $(\text{C}_1-\text{C}_6)\text{alkyl}$ ; or an N-heterocyclic ring optionally con-  
 taining 1 or 2 additional  $\text{N}(\text{R}_q)$ , S, or nonperoxide O,  
 wherein  $\text{R}_3$  is  $\text{H}(\text{C}_1-\text{C}_6)\text{alkyl}$ , phenyl, or benzyl;  
 $\text{R}_m$  and  $\text{R}_q$  are independently hydrogen,  $(\text{C}_1-\text{C}_6)\text{alkyl}$ ,  
 phenyl, benzyl, or  $(\text{C}_1-\text{C}_6)\text{alkenyl}$ ; or  $\text{R}_m$  and  $\text{R}_q$   
 together with the nitrogen to which they are attached  
 are a 3, 4, 5, or 6 membered heterocyclic ring;  
 $\text{R}_p$  is H or  $(\text{C}_1-\text{C}_6)\text{alkyl}$ ; and  
 $\text{R}_m$  and  $\text{R}_q$  are independently hydrogen,  $(\text{C}_1-\text{C}_6)\text{alkyl}$ ,  
 phenyl, benzyl, or  $(\text{C}_1-\text{C}_6)\text{alkenyl}$ ;  
 the compound is MER25;  
 or a pharmaceutically acceptable salt thereof.  
 10. The method of any one of claims 1, 2, 3, 4, 8, and 9  
 wherein Z is  $-(\text{CH}_2)_{3-5}$ ,  $-\text{O}-$ ,  $-\text{OCH}_2-$ ,  $-\text{CH}_2\text{O}-$ ,  
 $-\text{C}(=\text{O})\text{O}-$ ,  $-\text{N}(\text{R}_q)-$ , or a covalent bond.  
 11. The method of any one of claims 1, 2, 3, 4, 8, and 9  
 wherein Z is  $-\text{O}-$ ,  $-\text{OCH}_2-$ ,  $-\text{CH}_2\text{O}-$ ,  $-\text{C}(=\text{O})$   
 $\text{O}-$ , or  $-\text{N}(\text{R}_q)-$ .  
 12. The method of any one of claims 1, 2, 3, 4, 8, and 9  
 wherein  $\text{R}^6$  is not phenyl or phenyl substituted by 1 or 2  $\text{V}$ .  
 13. The method of any one of claims 1, 2, 3, 4, 8, and 9  
 wherein  $\text{R}^7$  is not phenyl or phenyl substituted by 1 or 2  $\text{V}$ .  
 14. The method of any one of claims 1, 2, 3, 4, 8, and 9  
 wherein  $\text{R}^8$  is not phenyl, or phenyl substituted by 1 or 2  $\text{V}$ .  
 15. A therapeutic method for preventing or treating a  
 condition or symptom associated with osteoporosis com-  
 prising administering to a mammal in need of such therapy,  
 an effective amount of a compound of formula VI:

(VII)



wherein

$\text{R}^6$  is  $(\text{C}_1-\text{C}_6)\text{alkyl}$ , or aryl, optionally substituted by 1, 2,  
 or 3  $\text{V}$ ;  
 $\text{R}^7$  is phenyl, optionally substituted by 1, 2, or 3  $\text{V}$ ; or  $\text{R}^7$   
 is  $(\text{C}_1-\text{C}_{12})\text{alkyl}$ , halo  $(\text{C}_1-\text{C}_{12})\text{alkyl}$ ,  $(\text{C}_3-\text{C}_6)$   
 cycloalkyl,  $(\text{C}_1-\text{C}_6)\text{alkylcyclo}(\text{C}_1-\text{C}_6)\text{alkyl}$ ,  $(\text{C}_3-\text{C}_6)$   
 cycloalkenyl, or  $(\text{C}_1-\text{C}_6)\text{alkyl}(\text{C}_3-\text{C}_6)\text{cycloalkenyl}$ ;  
 $\text{R}^8$  is hydrogen or phenyl, optionally substituted by 1, 2,  
 or 3  $\text{V}$ ;  
 $\text{R}^9$  is hydrogen, halo, aryl, aryl  $(\text{C}_1-\text{C}_6)\text{alkyl}$ , halo  
 $(\text{C}_1-\text{C}_{12})\text{alkyl}$ , cyano  $(\text{C}_1-\text{C}_{12})\text{alkyl}$ , or  $(\text{C}_1-\text{C}_{12})\text{alkyl}$ ,  
 wherein any aryl may optionally be substituted by 1, 2,  
 or 3  $\text{V}$ ; or  
 — is a single bond or is  $-\text{C}(\text{B})(\text{D})-$ , wherein B and D  
 are each independently hydrogen,  $(\text{C}_1-\text{C}_6)\text{alkyl}$ , or  
 halo;  
 $\text{V}$  is  $\text{OPO}(\text{H})_2$ ,  $(\text{C}_1-\text{C}_6)\text{alkyl}$ ,  $(\text{C}_1-\text{C}_6)\text{alkoxy}$ , mercapto,  
 $(\text{C}_1-\text{C}_6)\text{alkylthio}$ , halo, trifluoromethyl,  
 pentafluoroethyl, nitro,  $\text{N}(\text{R}_m)(\text{R}_q)$ , cyano,

trifluoromethoxy, pentafluoroethoxy, benzoyl, hydroxy,  $-(CH_2)_{0-4}C(=O)(C_1-C_6)alkyl$ ,  $-UC(=O)(C_1-C_6)alkyl$ , benzyl,  $-OSO_2(CH_2)_{0-4}CH_3$ ,  $-U(CH_2)_{1-4}COOR_p$ ,  $-(CH_2)_{0-4}COOR_p$ ,  $-U(CH_2)_{2-4}OR_p$ ,  $-(CH_2)_{0-4}OR_p$ ,  $U(CH_2)_{1-4}C(=O)R_q$ ,  $-(CH_2)_{0-4}C(=O)R_q$ ,  $-U(CH_2)_{1-4}R_s$ ,  $-(CH_2)_{0-4}R_s$ , or  $-U(CH_2)_{2-4}OC(=O)R_p$  wherein U is O, N(R<sub>m</sub>), or S; Z is  $-(CH_2)_{1-3}$ ,  $-O$ ,  $-OCH_2$ ,  $-CH_2O$ ,  $-C(=O)O$ ,  $-N(R_s)$ , or a covalent bond;

R<sub>3</sub> is amino, optionally substituted with one or two (C<sub>1</sub>-C<sub>6</sub>)alkyl; or an N-heterocyclic ring optionally containing 1 or 2 additional N(R<sub>i</sub>), S, or nonperoxide O, wherein R<sub>1</sub> is H(C<sub>1</sub>-C<sub>6</sub>)alkyl, phenyl, or benzyl;

R<sub>4</sub> and R<sub>5</sub> are independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, phenyl, benzyl, or (C<sub>1</sub>-C<sub>6</sub>)alkanoyl; or R<sub>4</sub> and R<sub>5</sub> together with the nitrogen to which they are attached are a 3, 4, 5, or 6 membered heterocyclic ring;

R<sub>6</sub> is H or (C<sub>1</sub>-C<sub>6</sub>)alkyl; and

R<sub>7</sub> and R<sub>8</sub> are independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, phenyl, benzyl, or (C<sub>1</sub>-C<sub>6</sub>)alkanoyl; the compound is MER25;

or a pharmaceutically acceptable salt thereof;

provided that R<sup>9</sup> is not methyl, ethyl, chloro, or bromo when Z is a covalent bond;

and provided that Z is not  $-CH_2-$  when R<sup>6</sup> is phenyl, substituted only at the 4-position with  $-O(CH_2)_{1-3}R_4$  wherein R<sub>4</sub> is pyrrolidine, piperidine, 4-morpholino, dimethylamino, diethylamino, or hexamethyleisimino; and provided that R<sup>7</sup> is not 2-chloroethyl when Z is a covalent bond; and R<sup>8</sup> is phenyl, substituted only at the 4-position with  $-O(CH_2)_{1-3}OH$ .

16. A therapeutic method for preventing or treating a condition or symptom associated with osteoporosis comprising administering to a mammal in need of such therapy, an effective amount of a compound of formula VI:

(VI)



wherein

R<sup>4</sup> is (C<sub>1</sub>-C<sub>6</sub>)alkyl, or aryl, optionally substituted by 1, 2, or 3 V<sub>i</sub>;

R<sup>7</sup> is phenyl, optionally substituted by 1, 2, or 3 V<sub>i</sub>; or R<sup>7</sup> is (C<sub>1</sub>-C<sub>12</sub>)alkyl, halo(C<sub>1</sub>-C<sub>12</sub>)alkyl, (C<sub>3</sub>-C<sub>6</sub>)cycloalkyl, (C<sub>1</sub>-C<sub>6</sub>)alkylcyclo(C<sub>1</sub>-C<sub>6</sub>)alkyl, (C<sub>3</sub>-C<sub>6</sub>)cycloalkenyl, or (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>3</sub>-C<sub>6</sub>)cycloalkenyl;

R<sup>6</sup> is hydrogen or phenyl, optionally substituted by 1, 2, or 3 V<sub>i</sub>;

R<sup>8</sup> is hydrogen, halo, aryl, aryl(C<sub>1</sub>-C<sub>6</sub>)alkyl, halo(C<sub>1</sub>-C<sub>12</sub>)alkyl, cyano(C<sub>1</sub>-C<sub>12</sub>)alkyl, or (C<sub>1</sub>-C<sub>12</sub>)alkyl, wherein any aryl may optionally be substituted by 1, 2, or 3 V<sub>i</sub>; or

— is a single bond or is  $-C(B)(D)-$ , wherein B and D are each independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, or halo;

V is OPO<sub>3</sub>H<sub>2</sub>, (C<sub>1</sub>-C<sub>6</sub>)alkyl, (C<sub>1</sub>-C<sub>6</sub>)alkoxy, mercapto, (C<sub>1</sub>-C<sub>6</sub>)alkylthio, halo, trifluoromethyl, pentafluoroethoxy, nitro, N(R<sub>m</sub>)(R<sub>n</sub>), cyano, trifluoromethoxy, pentafluoroethoxy, benzoyl, hydroxy,  $-(CH_2)_{0-4}C(=O)(C_1-C_6)alkyl$ ,  $-UC(=O)(C_1-C_6)alkyl$ , benzyl,  $-OSO_2(CH_2)_{0-4}CH_3$ ,  $-U(CH_2)_{1-4}COOR_p$ ,  $-(CH_2)_{0-4}COOR_p$ ,  $-U(CH_2)_{2-4}OR_p$ ,

alkyl, benzyl,  $-OSO_2(CH_2)_{0-4}CH_3$ ,  $-U(CH_2)_{1-4}COOR_p$ ,  $-(CH_2)_{0-4}COOR_p$ ,  $-U(CH_2)_{2-4}OR_p$ ,  $-(CH_2)_{0-4}OR_p$ ,  $-U(CH_2)_{1-4}C(=O)R_q$ ,  $-(CH_2)_{0-4}C(=O)R_q$ ,  $-U(CH_2)_{1-4}R_s$ ,  $-(CH_2)_{0-4}R_s$ , or  $-U(CH_2)_{2-4}OC(=O)R_p$  wherein U is O, N(R<sub>m</sub>), or S;

Z is  $-(CH_2)_{1-3}$ ,  $-O$ ,  $-OCH_2$ ,  $-CH_2O$ ,  $-C(=O)O$ ,  $-N(R_s)$ , C=O, or a covalent bond;

R<sub>4</sub> is amino, optionally substituted with one or two (C<sub>1</sub>-C<sub>6</sub>)alkyl; or an N-heterocyclic ring optionally containing 1 or 2 additional N(R<sub>i</sub>), S, or nonperoxide O, wherein R<sub>1</sub> is H(C<sub>1</sub>-C<sub>6</sub>)alkyl, phenyl, or benzyl;

R<sub>4</sub> and R<sub>5</sub> are independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, phenyl, benzyl, or (C<sub>1</sub>-C<sub>6</sub>)alkanoyl; or R<sub>4</sub> and R<sub>5</sub> together with the nitrogen to which they are attached are a 3, 4, 5, or 6 membered heterocyclic ring;

R<sub>6</sub> is H or (C<sub>1</sub>-C<sub>6</sub>)alkyl; and

R<sub>7</sub> and R<sub>8</sub> are independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, phenyl, benzyl, or (C<sub>1</sub>-C<sub>6</sub>)alkanoyl;

the compound is MER25;

or a pharmaceutically acceptable salt thereof;

provided that Z is not a covalent bond when R<sup>6</sup> is methyl, ethyl, chloro, or bromo;

and provided that Z is not  $-CH_2-$  when R<sup>6</sup> is phenyl, substituted only at the 4-position with  $-O(CH_2)_{1-3}R_4$  wherein R<sub>4</sub> is pyrrolidine, piperidine, 4-morpholino, dimethylamino, diethylamino, or hexamethyleisimino; and provided that R<sup>7</sup> is not halo(C<sub>1</sub>-C<sub>12</sub>)alkyl when Z is a covalent bond; and R<sup>8</sup> is phenyl, substituted only at the 4-position with  $-O(CH_2)_{1-3}OH$ .

17. A therapeutic method for preventing or treating a condition or symptom associated with osteoporosis comprising administering to a mammal in need of such therapy, an effective amount of a compound of formula VI:

(VII)



wherein

R<sup>4</sup> is (C<sub>1</sub>-C<sub>6</sub>)alkyl, or aryl, optionally substituted by 1, 2, or 3 V<sub>i</sub>;

R<sup>7</sup> is phenyl, optionally substituted by 1, 2, or 3 V<sub>i</sub>; or R<sup>7</sup> is (C<sub>1</sub>-C<sub>12</sub>)alkyl, halo(C<sub>1</sub>-C<sub>12</sub>)alkyl, (C<sub>3</sub>-C<sub>6</sub>)cycloalkyl, (C<sub>1</sub>-C<sub>6</sub>)alkylcyclo(C<sub>1</sub>-C<sub>6</sub>)alkyl, (C<sub>3</sub>-C<sub>6</sub>)cycloalkenyl, or (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>3</sub>-C<sub>6</sub>)cycloalkenyl;

R<sup>6</sup> is hydrogen or phenyl, optionally substituted by 1, 2, or 3 V<sub>i</sub>;

R<sup>8</sup> is hydrogen, halo, aryl, aryl(C<sub>1</sub>-C<sub>6</sub>)alkyl, halo(C<sub>1</sub>-C<sub>12</sub>)alkyl, cyano(C<sub>1</sub>-C<sub>12</sub>)alkyl, or (C<sub>1</sub>-C<sub>12</sub>)alkyl, wherein any aryl may optionally be substituted by 1, 2, or 3 V<sub>i</sub>; or

— is a single bond or is  $-C(B)(D)-$ , wherein B and D are each independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, or halo;

V is OPO<sub>3</sub>H<sub>2</sub>, (C<sub>1</sub>-C<sub>6</sub>)alkyl, (C<sub>1</sub>-C<sub>6</sub>)alkoxy, mercapto, (C<sub>1</sub>-C<sub>6</sub>)alkylthio, halo, trifluoromethyl, pentafluoroethoxy, nitro, N(R<sub>m</sub>)(R<sub>n</sub>), cyano, trifluoromethoxy, pentafluoroethoxy, benzoyl, hydroxy,  $-(CH_2)_{0-4}C(=O)(C_1-C_6)alkyl$ ,  $-UC(=O)(C_1-C_6)alkyl$ , benzyl,  $-OSO_2(CH_2)_{0-4}CH_3$ ,  $-U(CH_2)_{1-4}COOR_p$ ,  $-(CH_2)_{0-4}COOR_p$ ,  $-U(CH_2)_{2-4}OR_p$ ,

$-(CH_2)_{0.4}OR_p$ ,  $-U(CH_2)_{1.4}C(=O)R_k$ ,  $-(CH_2)_{0.4}C(=O)R_k$ ,  $-U(CH_2)_{1.4}R_k$ ,  $-(CH_2)_{0.4}R_k$ , or  $-U(CH_2)_{2.4}OC(=O)R_p$ ; wherein U is O,  $N(R_m)$ , or S; Z is  $-O-$ ,  $-OCH_2-$ ,  $-CH_2O-$ ,  $-C(=O)O-$ , or  $-N(R_m)-$ ;

$R_k$  is amino, optionally substituted with one or two  $(C_1-C_6)$ alkyl; or an N-heterocyclic ring optionally containing 1 or 2 additional  $N(R_1)$ , S, or nonperoxide O, wherein  $R_1$  is  $H(C_1-C_6)$ alkyl, phenyl, or benzyl;

$R_m$  and  $R_n$  are independently hydrogen,  $(C_1-C_6)$ alkyl, phenyl, benzyl, or  $(C_1-C_6)$ alkanoyl; or  $R_m$  and  $R_n$  together with the nitrogen to which they are attached are a 3, 4, 5, or 6 membered heterocyclic ring;

$R_p$  is H or  $(C_1-C_6)$ alkyl; and

$R_m$  and  $R_n$  are independently hydrogen,  $(C_1-C_6)$ alkyl, phenyl, benzyl, or  $(C_1-C_6)$ alkanoyl; the compound is MER25;

or a pharmaceutically acceptable salt thereof.

18. A therapeutic method for preventing or treating a condition or symptom associated with osteoporosis comprising administering to a mammal in need of such therapy, an effective amount of a compound of formula VI:



wherein

$R^6$  is  $(C_1-C_6)$ alkyl, or aryl, optionally substituted by 1, 2, or 3 V;

$R^7$  is phenyl, optionally substituted by 1, 2, or 3 V; or  $R^7$  is  $(C_1-C_{12})$ alkyl, halo $(C_1-C_{12})$ alkyl,  $(C_3-C_6)$ cycloalkyl,  $(C_1-C_6)$ alkylcyclo $(C_3-C_6)$ alkyl,  $(C_3-C_6)$ cycloalkenyl, or  $(C_1-C_6)$ alkyl $(C_3-C_6)$ cycloalkenyl;

$R^8$  is hydrogen or phenyl, optionally substituted by 1, 2, or 3 V;

$R^9$  is hydrogen, halo, aryl, aryl $(C_1-C_6)$ alkyl, halo $(C_1-C_{12})$ alkyl, cyano $(C_1-C_{12})$ alkyl, or  $(C_1-C_{12})$ alkyl, wherein any aryl may optionally be substituted by 1, 2, or 3, V; or

$-$  is a single bond or is  $-C(B)(D)-$ , wherein B and D are each independently hydrogen,  $(C_1-C_6)$ alkyl, or halo;

V is  $OPO_3H_2$ ,  $(C_1-C_6)$ alkyl,  $(C_1-C_6)$ alkoxy, mercapto,  $(C_1-C_6)$ alkylthio, halo, trifluoromethyl, pentafluoroethyl, nitro,  $N(R_m)(R_n)$ , cyano, trifluoromethoxy, pentafluoroethoxy, benzoyl, hydroxy,  $-(CH_2)_{0.4}C(=O)(C_1-C_6)$ alkyl,  $-UC(=O)(C_1-C_6)$ alkyl, benzyl,  $-OSO_2(CH_2)_{0.4}CH_3$ ,  $-U(CH_2)_{1.4}COOR_p$ ,  $-(CH_2)_{0.4}COOR_p$ ,  $-U(CH_2)_{2.4}OR_p$ ,  $-(CH_2)_{0.4}OR_p$ ,  $-U(CH_2)_{1.4}C(=O)R_k$ ,  $-(CH_2)_{0.4}C(=O)R_k$ ,  $-U(CH_2)_{2.4}R_k$ , or  $-U(CH_2)_{2.4}OC(=O)R_p$ ; wherein U is O,  $N(R_m)$ , or S;

Z is  $-(CH_2)_{1.5}-$ ,  $-O-$ ,  $-OCH_2-$ ,  $-CH_2O-$ ,  $-C(=O)O-$ ,  $-N(R_m)-$ ,  $C=O$ , or a covalent bond;

$R_k$  is amino, optionally substituted with one or two  $(C_1-C_6)$ alkyl; or an N-heterocyclic ring optionally containing 1 or 2 additional  $N(R_1)$ , S, or nonperoxide O, wherein  $R_1$  is  $H(C_1-C_6)$ alkyl, phenyl, or benzyl;

$R_m$  and  $R_n$  are independently hydrogen,  $(C_1-C_6)$ alkyl, phenyl, benzyl, or  $(C_1-C_6)$ alkanoyl; or  $R_m$  and  $R_n$  together with the nitrogen to which they are attached are a 3, 4, 5, or 6 membered heterocyclic ring;

$R_p$  is H or  $(C_1-C_6)$ alkyl; and

$R_m$  and  $R_n$  are independently hydrogen,  $(C_1-C_6)$ alkyl, phenyl, benzyl, or  $(C_1-C_6)$ alkanoyl;

the compound is MER25;

or a pharmaceutically acceptable salt thereof;

provided  $R^6$  is not phenyl or phenyl substituted by 1 or 2 V.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
CERTIFICATE OF CORRECTION

PATENT NO.: 6,117,911  
DATED: Sep. 12, 2000  
INVENTOR(S): Grainger et al.

It is certified that errors appear in the above-identified patent and that said Patent is hereby corrected as shown below:

- In column 41, line 58 add --  $\beta$  -- after "TGF-", therefor.
- In column 65, line 32 delete " $(CH_2)_{10}$ " and insert  $-(CH_2)_{10-4}$  --, therefor.
- In column 65, line 33 delete " $(CH_2)_{10}$ " and insert  $-(CH_2)_{10-4}$  --, therefor.
- In column 66, line 59 delete " $(C_1-C_{21})$ " and insert  $-(C_1-C_{12})$  --, therefor.
- In column 66, line 61 delete " $(C_{13}-C_{20})$ " and insert  $-(C_3-C_6)$  --, therefor.
- In column 67, line 52 delete " $R^1$ " and insert  $-R^1$  --, therefor.
- In column 68, line 7 delete " $-(CH_2)_{10}$ " and insert  $-(CH_2)_{10-4}$  --, therefor.
- In column 68, line 13 delete " $N(R_1)$ ," and insert  $-N(R_1)$  --, therefor.
- In column 69, line 9 delete " $N(R_1)$ ," and insert  $-N(R_1)$  --, therefor.
- In column 70, line 12 delete " $N(R_1)$ ," and insert  $-N(R_1)$  --, therefor.
- In column 71, line 12 delete " $N(R_1)$ ," and insert  $-N(R_1)$  --, therefor.

Signed and Sealed this  
First Day of May, 2001

Attest:



NICHOLAS P. GODICI

Attesting Officer

Acting Director of the United States Patent and Trademark Office



US005516528A

**United States Patent** [19]

Hughes et al.

[11] **Patent Number:** 5,516,528[45] **Date of Patent:** May 14, 1996[54] **DIETARY PHYTOESTROGEN IN ESTROGEN REPLACEMENT THERAPY**[75] Inventors: Claude L. Hughes, Mebane, N.C.;  
Edna C. Henley, St. Louis, Mo.;  
Thomas B. Clarkson, Clemmons, N.C.[73] Assignees: Wake Forest University,  
Winston-Salem, N.C.; Protein  
Technologies International, Inc., St.  
Louis, Mo.

[21] Appl. No.: 372,750

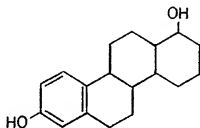
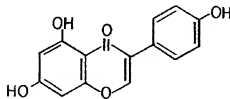
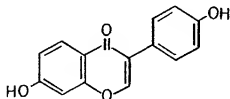
[22] Filed: Jan. 13, 1995

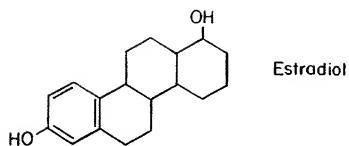
[51] Int. Cl.<sup>6</sup> ..... A61K 9/28; A61K 9/70;  
A61K 35/78; A61F 2/02[52] U.S. Cl. .... 424/464; 424/423; 424/449;  
424/451; 424/195.1; 514/182[58] Field of Search ..... 424/449, 451,  
424/464, 195.1, 423; 514/182[56] **References Cited****U.S. PATENT DOCUMENTS**

5,277,910 1/1994 Hidaugi ..... 424/195.1

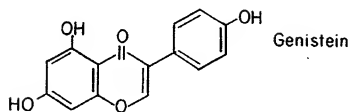
**OTHER PUBLICATIONS**Ursin et al., In: Doll et al., eds., *Cancer surveys—trends in cancer incidence and mortality*, Plainview NY: Cold Spring Harbor Laboratory Press, 241 (1994).Shimizu et al., *Br. J. Cancer* 63: 963 (1991).Coward et al., *J. Agr. Food Chem.* 41: 1961 (1993).Barnes et al., In Jacobs MM, ed., *Diet and Cancer: Markers, prevention and treatment*, New York: Plenum Press, 135 (1994).Mackiewicz et al., *J. Steroid Biochem.* 45: 399 (1993).Whitten et al., *Steroids* 59: 443 (1994).Anthony et al., *Circulation* 90: Abstract I-235, Oct. 1994.*Primary Examiner*—Carlos Azpuru  
*Attorney, Agent, or Firm*—Kevin M. Farrell**[57] ABSTRACT**

Disclosed is a pharmaceutical composition for oral delivery. The composition includes about 1–2 mg mammalian estrogen and about 25–100 mg phytoestrogen. Compositions of the type described above are utilized, for example, in a therapeutic regimen designed to reduce the risk of coronary heart disease and osteoporosis in postmenopausal women. This method comprises the oral administration of a composition comprising a mixture of estrogen and phytoestrogen, the dosages of mammalian estrogen and phytoestrogen being about 1–2 mg, about 25–100 mg, respectively.

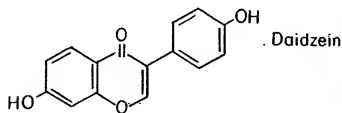
**19 Claims, 1 Drawing Sheet****Estradiol****Genistein****Daidzein**



Estradiol



Genistein



Daidzein

FIGURE 1

# 1

## DIETARY PHYTOESTROGEN IN ESTROGEN REPLACEMENT THERAPY

### GOVERNMENT SUPPORT

Work described herein was supported by grants from the National Institutes of Health and the United States Government has certain rights in the invention.

### BACKGROUND OF THE INVENTION

Coronary heart disease (CHD) is the leading cause of death in women, accounting for 36% of all deaths (approximately 380,000 deaths per year). CHD-related mortality is greater than breast and lung cancer mortality combined. Clinical studies have shown that estrogen replacement therapy (ERT) in the postmenopausal period may reduce morbidity and mortality by 50% or more.

Estrogen deprivation is also an important cause of postmenopausal osteoporosis. Osteoporosis affects about one-third to one-half of all postmenopausal women. Annually, 500,000 vertebral fractures occur. Nearly one-third of women over age 65 will suffer at least one vertebral fracture. Estrogen replacement therapy has been determined to substantially reduce the risk of osteoporosis.

In spite of the reports of substantial reduction in the incidence of both coronary heart disease and osteoporosis associated with estrogen replacement therapy, poor compliance with such therapy has prevented a major impact on women's health overall. Studies have found that the two principal disincentives for women to continue estrogen replacement therapy are fear of breast cancer, and the adverse effects of the progestin (i.e., the nuisance of continuing menstrual periods, cyclic depression, breast tenderness and symptoms like those of premenstrual syndrome) used to prevent endometrial carcinoma. Aside from the breast cancer fear, the other disincentives all relate to the need to use a cyclic or continuous progestin (i.e., medroxyprogesterone acetate) to prevent the risk of endometrial carcinoma.

### SUMMARY OF THE INVENTION

The subject invention relates to a pharmaceutical composition for oral delivery. The composition includes about 1-2 mg mammalian estrogen and about 25-100 mg phytoestrogen. In a preferred embodiment, the pharmaceutical composition is in pill or capsule form. The invention also encompasses transdermal or implant delivery systems calibrated to deliver estrogen and phytoestrogen within the previously stated ranges.

Preferably the estrogen is estradiol, and the phytoestrogen is a phytoestrogen which functions as an estrogen antagonist in breast and uterine tissue. Members of the isoflavone class of phytoestrogens (e.g., genistein and daidzein) are particularly preferred.

Compositions of the type described above are utilized, for example, in a therapeutic regimen designed to reduce the risk of coronary heart disease and osteoporosis in postmenopausal women. This method comprises the oral administration of a composition comprising a mixture of estrogen and phytoestrogen, the dosages of mammalian estrogen and phytoestrogen being about 1-2 mg. about 25-100 mg, respectively.

# 2

## BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 is a diagram showing the chemical structures of the soy phytoestrogens genistein (4',5,7-trihydroxyisoflavone) and daidzein (4',7-dihydroxyisoflavone) compared with estradiol of mammalian origin.

### DETAILED DESCRIPTION OF THE INVENTION

Nearly 70 years ago, it was reported that certain plants could induce estrus in animals. Subsequently, over 300 plants have been found to possess estrogenic activity (see e.g., Bradbury and White, *Vitamin Horm.* 12:207 (1954), and Farnsworth et al., *J. Pharm. Sci.* 64:717 (1954)). These compounds have been given the general name of "phytoestrogens" and represent several chemical classes. Similarities in the molecular structure of phytoestrogens facilitate binding to the estrogen receptor. An important class of phytoestrogens is the isoflavone class. The chemical structures of the soy isoflavones genistein (4',5,7-trihydroxyisoflavone) and daidzein (4',7-dihydroxyisoflavone) are compared with estradiol of mammalian origin in FIG. 1. Phytoestrogens have been shown to exhibit mixed estrogen agonist-antagonist properties which are organ-specific in vivo (Seichell, In McLachlan, Ed., *Estrogens in the Environment*, New York, Elsevier, pp 69-85 (1985)).

Soybeans are a particularly important source of phytoestrogen. Several hundred varieties or cultivars of soybeans exist, and their phytoestrogen content can vary from 50 mg/100 g to 300 mg/100 g. In addition, given the high levels of consumption of soy protein by certain Oriental cultures, there is a substantial body of relevant epidemiologic evidence. For example, there is epidemiologic evidence that phytoestrogens are associated with lower risk of development of breast and uterine cancer. Oriental women have lower rates of breast cancer compared to Americans. For example, in 1985, the age-adjusted breast cancer incidence in the United States among women aged 35 to 74 was 211.9 per 100,000 women, compared to 69.7 per 100,000 in Japan (Ursin et al., In: Doll et al., eds, *Cancer surveys—trends in cancer incidence and mortality*, Plainville, N.Y.: Cold Spring Harbor Laboratory Press, 241 (1994)). Additionally, Japanese women who have emigrated to the United States are at higher risk of breast cancer than those who remain in Japan, and the risk of cancer in their offspring approaches that of other US-born women (Shimizu et al., *Br. J. Cancer* 63:963 (1991)). Oriental women consume 30 to 50 times more soy products than American women (Coward et al., *J. Agr. Food Chem.* 41:1961 (1993)); as a result, differences in urinary excretion of phytoestrogens between Oriental women and American women are striking (Oriental: 2,000-3,000 nmol/24 hour of genistein and daidzein; American: 30-40 nmol/24 hour). Experimental evidence for lower breast cancer incidence associated with dietary phytoestrogens in soybeans has also been reported (Barnes et al., In Jacobs MM, ed., *Diet and Cancer: Markers, prevention and treatment*, New York: Plenum Press, 135 (1994)). Specifically, dietary soy protein preparations enriched with phytoestrogens inhibited mammary tumors in rats treated with 7,12-dimethyl-benz[a]anthracene compared to rats fed low-phytoestrogen soy protein preparations.

The differences in endometrial cancer incidence between North American (Canadian) and Asian women (Japanese) are also noteworthy. For example, the age-specific endometrial cancer incidence in 1985 increased with age in North America. In contrast, there was no age-associated increase

among Japanese women and incidence was lower for each age group compared to North Americans.

The increasingly frequent suggestion that phytoestrogens may protect against development of coronary artery atherosclerosis (CAA) and CHD is based on the evidence that endogenous estrogen protects premenopausal women from CHD relative to men of the same age, that loss of ovarian hormones accounts for the progressing CHD of postmenopausal women, and that estrogen replacement decreases CHD risk among postmenopausal women. That phytoestrogens may be cardioprotective is supported by the low rates of CHD among Oriental people compared to Westerners, and the low rates of CHD among vegetarians compared to omnivorous people, although differences in dietary fat and cholesterol confound these comparisons.

In addition to the epidemiologic evidence, recent experimental observations have suggested that phytoestrogens may protect against the development of CAA. For example, studies have shown that male casein-fed rats had significantly higher total plasma cholesterol (TPC) and low density lipoprotein cholesterol (LDL-C) concentrations than soy protein-fed rats. When soybean phytoestrogens were added to casein, the animals had LDL-C concentrations similar to the soy protein-fed group.

In premenopausal female monkeys fed soy protein enriched in phytoestrogens, TPC concentrations were significantly lower and HDL-C concentrations were higher among phytoestrogen-enriched soy protein-fed animals. Furthermore, among surgically postmenopausal female monkeys, HDL-C concentrations were higher (50 versus 67 mg/dl), while TPC concentrations were similar between phytoestrogen-enriched and low-phytoestrogen soy-fed groups.

Finally, among the phytoestrogens, only genistein (the principal phytoestrogen of soybeans) has been the subject of numerous cellular/molecular biologic studies suggesting its potential beneficial effects of several key aspects of CAA, restenosis and arterial function. A summary of those observations is shown in Table 1.

TABLE 1

Genistein Effects	Reference
Inhibits interleukin-2 and leukotriene B <sub>4</sub> production by mononuclear cells	Ashur et al., Clin. Immunol. Immunopathol. 59:579 (1991)
Inhibits platelet-derived growth factor + smooth muscle cell proliferation	Fujio et al., Biochem. Biophys. Res. Commun. 195: 79 (1993)
Inhibits aortic fibroblast mRNA expression	Hosoi et al., Circ. Res. 73:689 (1993)
Inhibits PDGF- $\alpha$ and c-myc proto-oncogene expression in aortic SMC	Vidalens et al., Biochem. Biophys. Res. Commun. 188:1198 (1992)
Suppresses tumor necrosis factor and IL-1 secretion by monocytes and T-lymphocytes	See et al., Infect. Immun. 60:3456 (1992)
Inhibits superoxide anion production by stimulated neutrophils	Urtum et al., Arch. Biochem. Biophys. 294:271 (1992)
Inhibits $\alpha$ -lipoic and $\alpha$ -lipoic expression by vascular SMC	Zerlin et al., Oncogene 6:219 (1991)
Increases nitric oxide expression by macrophages	Dong et al., J. Immunol. 151:2717 (1993)
Inhibits endothelial production by endothelial cells	Ito et al., Diabetes 42:351 (1993)
Inhibits calcium channel currents in vascular smooth muscle cells	Wijetunge et al., Biochem. Biophys. Res. Commun. 189:1620 (1992)
Inhibits thrombin-induced platelet aggregation	Asahi et al., FEBS Lett 309:10 (1992)

TABLE 1-continued

Genistein Effects	Reference
Inhibits platelet-activating factor	Kawafuta et al., J. Immunol. 151:637 (1993)
Inhibits thromboxane-induced platelet aggregation	McNicol et al., Prostaglandins Leukot. Essent. Fatty Acids 48:379 (1992)

In addition to the evidence discussed above in connection with CHD and CAA, there is also epidemiologic and experimental evidence that phytoestrogen can prevent postmenopausal bone loss and osteoporosis. With respect to the epidemiologic evidence, differences in hip fracture incidence between U.S. women (low phytoestrogen consumers) and Asian women (high phytoestrogen consumers) can not be explained by the usual relationships with calcium consumption. More specifically, the per capita calcium consumption of calcium (mg/day) of U.S. women exceeds 1,000 mg/day, whereas the per capita calcium consumption of Asian women is only slightly above 500 mg/day. However, the incidence of hip fractures per 100,000 women in the U.S. is about 100, whereas the Asian population incidence is only about 22 per 100,000 women.

With regard to the experimental data, a recent report discloses that a low dose, but not a high dose of genistein was equivalent to conjugated equine estrogen (CEE) in maintaining bone mass in ovariectomized rats. Several reports have provided further evidence for a bone-protective effect of genistein. These reports focused on a structurally similar compound, ipriflavone, which is a synthetic 7-isopropoxyisoflavone (Gambacciani et al., *J. Endocrinol. Invest.* 16:333 (1993); Melis et al., In Christiansen C., Riis B., eds, *Proceedings, 4th International Symposium on Osteoporosis and Consensus Development Conference*: 460 (1993); Passeri et al., In Christiansen C., Riis B., eds, *Proceedings, 4th International Symposium on Osteoporosis and Consensus Development Conference*: 463 (1993); Agnolucci et al., In Christiansen C., Riis B., eds, *Proceedings, 4th International Symposium on Osteoporosis and Consensus Development Conference*: 467 (1993).

The epidemiologic and experimental results discussed above are consistent with a hypothesis that dietary phytoestrogen represents a critical element in the diet of Oriental women which is, at the very least, non-antagonistic of the positive post-menopausal effects of estrogen in bone and cardiac tissue, while functioning antagonistically in breast and uterine tissue. If true, this hypothesis suggests that a dietary regimen designed to produce serum levels of estrogen and phytoestrogen mimicking those of premenopausal Oriental women represents a better alternative to current estrogen replacement therapy. More specifically, the known risks of increased incidence of breast cancer associated with hormone replacement therapy would be reduced by the antagonist effects of phytoestrogen in breast tissue. Similarly, in uterine tissue, the antagonistic effects of phytoestrogen would result in a reduction in the risk of endometrial cancer relative to conventional estrogen replacement therapy. Furthermore, this estrogen antagonistic effect in uterine tissue would obviate the need for the co-administration of progestin. As discussed previously, the side effects of progestin have been identified as a major disincentive with respect to compliance with an estrogen therapy regimen.

Having said this, however, there is contrary experimental evidence which must be considered. More specifically, Wilcox et al. (*Br. Med. J.* 301:905 (1990)) reported increases in

vaginal cell proliferation among postmenopausal women consuming soybean phytoestrogens for 6 weeks. In addition, Markiewicz et al. (*J. Steroid Biochem.* 45:399 (1993)) demonstrated experimentally that the soy isoflavone genistein exhibited an estrogen effect on endometrial cancer cells in an *in vitro* bioassay. These reports are in conflict with the epidemiological and experimental data discussed above.

The subject invention is based on Applicants' discovery that contrary indications in the prior art with respect to vaginal epithelial cell proliferation are not borne out in a carefully controlled study in an animal model system. The animal model selected for initial study was the cynomolgus macaque. Cynomolgus macaque females have physiological and reproductive characteristics similar to those of women (Mahoney CJ, *J. Reprod. Fert.* 21:153 (1970); MacDonald GJ, *Fertil. Steril.* 22:373 (1971); Jewett DA and Dukelow WK, *J. Reprod. Fert.* 31:287 (1972)). Like premenopausal women, premenopausal cynomolgus macaque females have significantly higher plasma concentrations of HDL-C than their male counterparts and gender differences in the extent of CAA are like those of human beings during the reproductively active years (Hamm et al., *Atherosclerosis* 48:221 (1983)).

As shown in Example 1, the soy isoflavone genistein does not act as an estrogen on the vaginal epithelium. The statistically significant data reported in Example 1 supports the epidemiological and experimental results which indicate that a diet which mimics the diet of Oriental women with respect to isoflavone consumption will reduce the known risks of increased incidence of breast cancer associated with hormone replacement therapy by virtue of the antagonistic effects of phytoestrogen in breast tissue. The data also supports the epidemiological and experimental results which indicate that a diet which mimics the diet of Oriental women with respect to isoflavone consumption will reduce the risk of endometrial cancer by virtue of the antagonistic effects of isoflavone resulting in a reduction in the risk of endometrial cancer relative to conventional estrogen replacement therapy. Furthermore, this estrogen antagonistic effect in uterine tissue would obviate the need for the co-administration of progestin. As discussed previously, the side effects of progestin have been identified as a major disincentive with respect to compliance with an estrogen therapy regimen.

Thus, in one aspect the invention relates to a pharmaceutical composition for oral delivery comprising about 1-2 mg mammalian estrogen and about 25-100 mg phytoestrogen. This composition would be administered orally to postmenopausal women. Preferably, the composition would be formulated in pill or capsule form using conventional manufacturing techniques. The preferred mammalian estrogen is *estrone-1,3,5(10)-triene-3,17 $\beta$ -diol*, commonly known as estradiol (e.g., Estrace®; Mead Johnson). The preferred phytoestrogen is selected from the isoflavonoid group. In particular, the isoflavonoids genistein and daidzein have been discussed previously. Transdermal and implant delivery systems calibrated to deliver about 1-2 mg estrogen and about 25-50 mg phytoestrogen/day are also encompassed by the present invention.

The administration of about 1-2 mg mammalian estrogen and about 25-100 mg phytoestrogen to postmenopausal women on a daily basis will serve to decrease the risk of osteoporosis and coronary heart disease, without the need for a co-administered progestin. In light of the fact that the side effects of progestin have been identified as one of the two major disincentives for compliance with estrogen replacement therapy, the present invention represents an estrogen replacement therapy regimen which will meet with

increased acceptance and compliance by women. The net effect of increased compliance will be a decrease in coronary heart disease and osteoporosis in postmenopausal women.

## EXAMPLES

### Example 1

Cynomolgus macaques are similar to women in many aspects of their reproductive physiology. Their endometrial responses to endogenous and exogenous hormones parallel those of women. In this study data from 2 ongoing research projects was used to determine the estrogenic effects of conjugated equine estrogens (CEE), Medroxyprogesterone (MPA), soybean estrogens (SBE) and Tamoxifen on the vaginal epithelium of surgically postmenopausal cynomolgus macaques. In the first study (study one), 40 surgically postmenopausal adult female cynomolgus macaques were randomized to either a control group (OVX) or one of two dietary treatments: CEE at a dose equivalent on a caloric basis to 0.625 mg/woman/day; or SBE at 1.27 mg genistein per g protein. In the second study (study two), 116 surgically postmenopausal adult female cynomolgus macaques were randomized into 5 dietary treatment groups. The five groups were control (OVX), CEE, MPA, CEE+MPA and Tamoxifen. CEE was given at the same dose as in study one and MPA was given at a dose equivalent on a caloric basis to 2.5 mg/woman/day to the MPA and MPA+CEE groups. Tamoxifen was given at a dose equivalent to a woman's dose of 20 mg per day.

Blood samples for study one were collected between May and June 1994 after the animals had been on the treatment for 4 to 6 months. Animals in study two had 5 to 8 blood samples collected over a period of two years on the treatments. Plasma estradiol levels were determined for animals in study one and plasma estradiol and estrone levels were determined for animals in study two to verify the treatments. Vaginal cytology smears were taken after 4 to 6 months of treatment for study one and after 2 years for study two. Maturation Index (MI) was determined using the superficial, intermediate and parabasal cell percentages. Statistical analysis was carried out using ANOVA. For study one the mean MI was 63.3, 66.1 and 98.2 for the OVX (control), SBE and CEE groups respectively. The mean estradiol levels over the treatment period were OVX=3.8, SBE=0, and CEE=147.1. Mean MI and plasma estradiol was significantly greater for animals on CEE compared to the controls or SBE fed animals. The SBE group was not significantly different from the controls. In study two, the mean MI were OVX (control)=67.9, CEE=96.3, MPA=57.8, CEE+MPA=74.4 and Tamoxifen=81.9. The CEE and Tamoxifen groups had significantly higher MI than the other groups but were not different from each other. The mean estradiol levels were OVX=7.0, CEE=107.3, MPA=10.6, CEE+MPA=109.6 and Tamoxifen=6.0. The CEE and CEE+MPA had significantly higher estradiol than the other groups. The estrone results were similar to the estradiol results with CEE and CEE+MPA groups having significantly higher levels than the other groups. These results demonstrate that CEE has estrogenic effects on the vaginal epithelium partly mediated through causing an increase in circulating levels of estradiol and estrone. Tamoxifen, an estrogen antagonist in breast tissue, has estrogenic effects on the vaginal epithelium. SBE containing genistein, does not act as an estrogen on the vaginal epithelium. MPA neutralizes the effects of CEE and has no estrogenic effects on the vaginal epithelium.

To evaluate the effect of dietary soy among postmenopausal women, ninety-seven women were randomly assigned (in approximately a 3:1 ratio) to a soy diet group after a two-week period when baseline measurements were taken. During the four weeks after randomization, the soy diet group ate daily portions of soy foods (provided by the study) as a substitute for approximately one third of their caloric intake. Members of the control group were instructed to eat "as usual" during the dietary intervention period. All participants were instructed to maintain a stable body weight (weight was recorded daily at home, and four times at a medical clinic). The following markers of estrogenicity were measured at baseline and again at the end of the dietary intervention: serum LH, serum FSH, serum SHBG, and cytology of the vaginal epithelium as reflected by the maturation index or percent superficial cells in vaginal smears. The concentrations of serum estradiol and urinary soy estrogens (urinary daidzein, genistein, and equol) were also measured at baseline and at the end of the dietary intervention period. All laboratory personnel were blinded to the time of collection (pre-diet or end-of-diet) and the treatment status (soy-supplemented diet or control) of all samples assayed. The study began with a pilot phase (n=8 women) and was then completed in two separate sessions, one in the fall (n=40 women) and one in the spring (n=49).

Study participants were volunteers who were recruited from the three-county area around Research Triangle Park, N.C. Recruitment was conducted through newspaper advertisements, fliers at restaurants and health food stores, radio announcements, and specific outreach to vegetarian organizations including Seventh Day Adventist groups. Criteria for entry were: 65 years of age or younger, at least two years past last menses, no use of estrogen replacement therapy or antibiotics in the preceding six months, no use of prescription drugs known to affect outcome measures, e.g., corticosteroids. An intensive participation schedule was required of each woman including weekly meetings, daily first-morning urine collection, four 24-hour urine collections, and four clinic visits to have blood drawn. Each woman received \$50/week to compensate for time and travel expenses. This study was approved by the Human Subjects Review Committee at the National Institute of Environmental Health Sciences.

Before randomization the women completed an extensive self-administered questionnaire regarding demographic information, lifestyle factors (exercise, cigarette smoking, coffee drinking, alcohol use), reproductive history, recent use of prescription and nonprescription drugs, and an adaptation of the "Health Habits and History Questionnaire" (Block et al., *Am. J. Epidemiol.* 124:453 (1986)) that collects information about dietary habits during the prior year. In addition, women completed a short daily questionnaire on which they recorded their weight, alcohol consumption, cigarette smoking, illnesses, prescription and nonprescription drug use, a set of scaled questions regarding how they felt that day, and (for those in the soy diet group) a record of soy food intake. After the diet intervention period they also filled out a short followup questionnaire to assess any major changes during the study in exercise patterns and (for the control group) diet.

After the first week in the study, daily first-morning urine specimens were collected and frozen at home. They were delivered to study personnel weekly in coolers with freezer packs to keep contents frozen. A 24-hour urine specimen was collected on the same day as the clinic appointment,

starting with the second void of the day and ending with the first-morning void (which was placed in a separate container) on the following day. The 24-hour specimens were kept in coolers with previously frozen freezer packs and delivered to study personnel at the end of the 24-hour collection period. Aliquots were frozen and stored at -20°C.

The major daily soy food was a main dish made from whole soybeans or texturized vegetable protein (dried defatted soybean flour). The whole soybeans were a single variety, organically grown, and purchased in a single batch. The texturized vegetable protein (TVP) was purchased in 50 lb. bags from a local health food store. Soy splits (dried soybeans), also from the local health food store, were provided as a daily snack. The soybeans, TVP, and soy splits were analyzed for daidzein and genistein by HPLC-mass spectrometry, as described previously (Setchell et al., *Gastroenterology* 93:225 (1987)). The daily intake of soy consisted of 38 gms of dry TVP (2.1 mg/g daidzein, 0.6 mg/g genistein) or 114 gms of dry whole soybeans (0.7 mg/g daidzein, 0.2 mg/g genistein). In addition, women ate 25 gms of soy splits daily (1.8 mg/g daidzein, 0.7 mg/g genistein). Thus, daily intake of isoflavones was 165 mg/day. This is approximately equivalent on a molar basis to 0.3 mg/day of conjugated steroidal estrogen assuming that the estrogenic activity of the phytoestrogens is about 0.1% that of conjugated estrogen.

Participants visited one of four medical clinics four times during the study: twice in the pre-diet period, and at the end of the diet period. All appointments were scheduled between 8:00 a.m. and 10:00 a.m., and women were instructed to fast from midnight the previous night until after their appointment. At each clinic appointment the women were weighed, and blood was drawn four times at twenty-minute intervals via venipuncture. Blood was centrifuged and equal aliquots of serum from each sample were pooled and stored at -20°C. The pooled serum sample was used for assays in order to reduce the variability caused by the pulsatile release of LH and FSH from the pituitary. At the second pre-diet and final visits, samples of vaginal epithelial cells were taken from the left and right midlateral vaginal walls. Clinical personnel collected cells by making 5-10 scraping strokes with vaginal spatulae. A separate slide was prepared for each wall. Study personnel assisted and immediately sprayed the slides with fixative containing alcohol and polyethylene glycol to preserve cells and prevent drying.

Urinary phytoestrogens (daidzein, genistein, and equol) were measured simultaneously in 5 ml. aliquots of each urine specimen. Isoflavones were extracted from urine by solid-phase extraction after addition of an internal standard 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\alpha$ -diol (5  $\mu$ g). Conjugates were hydrolyzed with  $\beta$ -glucuronidase and sulfatase enzymes. Unconjugated estrogens were extracted by liquid-solid extraction, and phenolic compounds were separated from neutral steroid hormone metabolites using an anion exchange gel, triethylaminohydroxypropyl Sephadex LH-20. Trimethylsilyl ethers were prepared, separated by gas chromatography on a DB-1 capillary column, and quantified by mass spectrometry using selected ion monitoring (Setchell et al., *Am. J. Clin. Nutr.* 40:569 (1984)).

Urinary concentrations of soy isoflavones were measured to demonstrate compliance with the diet and to provide a crude measure of phytoestrogen dose for each participant. Earlier work (Setchell et al., *Am. J. Clin. Nutr.* 40:569 (1984)) had shown that urinary excretion levels increased by the third day of a soy-supplemented diet, remained high but quite variable during the diet, and returned to low baseline levels by the third or fourth day after cessation of the diet.

To minimize the effect of day-to-day variations in urinary isoflavone levels, first morning urine samples from before the diet (6 ml. aliquots from each of the seven days before randomization) and during the diet period (2 ml. aliquots from each day of the last three weeks of the diet) were pooled prior to phytoestrogen measurement in the pooled sample. Concentrations were expressed relative to the creatinine concentration in the pooled sample. A pilot study of 20 paired specimens had been conducted to measure phytoestrogen concentrations in 24-hour and first-morning urine specimens from the same 24-hour period to verify that first-morning urine specimens (corrected for creatinine) were valid indicators of total urinary excretion.

LH, FSH, SHBG, and estradiol concentrations in sera were measured with commercial kits. Time-resolved fluorimmunoassays for LH, FSH and SHBG were performed with the appropriate LKB-Wallac DELFIA kits (Electronuclear, Inc., Columbia, Md.). Estradiol was measured by radioimmunoassay (Leeco Diagnostics, Inc., Southfield, Mich.). Samples were analyzed in duplicate and the geometric means of the duplicates were used for analysis. All samples from an individual woman were assayed together. For all analyses, the intra-assay coefficient of variation was <5% and the inter-assay coefficient of variation was <10% based on quality control standards.

All vaginal smear slides were read by a single trained technician. Specimens from each wall were read separately, and the technician was not aware of which slides were paired. From each slide two hundred cells were examined to determine the percentage of parabasal, intermediate, and superficial cells (Hammond, D.O., *Clin. Obstet. Gynecol.* 4: 49 (1977)). The values from the two walls were averaged to give a single value for each woman. Fifty-one (14%) of the 364 slides had too few cells to count and were not included in the calculations. This resulted in four women with no vaginal smear data and 34 women with vaginal smear data based on only one wall for at least one of the time periods. A maturation index was calculated as the percent superficial cells plus half the percent intermediate cells.

The outcome measures in this study were changes during the four-week diet period in serum FSH, LH, SHBG, and vaginal cell maturation index. For maturation index, the change variable was the difference between the end-of-diet value and the baseline value. For FSH, LH, and SHBG, the change variable was the difference in the natural logarithms of the end-of-diet and baseline values, which is equivalent to the logarithms of the ratio: end-of-diet divided by baseline. FSH, LH, and SHBG concentrations were logarithmically transformed before analysis because the transformed values better met normality and equal variance assumptions. Baseline concentrations were estimated as the geometric mean of two pre-diet values.

For each of the four dependent variables (change in FSH, LH, SHBG, and maturation index), tests were conducted for the effect of dietary intervention by including treatment as a term in a basic model that also included season of study, and clinic that the woman attended. Thus, the null hypothesis was that the mean change for the soy diet group was not different from the mean change in the control group. Change in serum estradiol level (difference in natural logarithms of end-of-diet and baseline concentrations), change in weight, and age (by chance, controls were younger on average than women in the soy diet group, though not significantly so) were also added one at a time to the basic model to adjust for possible effects of these factors. Changes in vaginal cytology were examined further by focussing analyses on the percent superficial cells, the cells considered most

indicative of estrogen stimulation (Blair, O. M., *Gynecologic Endocrinology*: 159 (1987)). Because only 27 women exhibited a change in superficial cells during the study (most remained at 0%), ordinal logistic regression with three levels of the dependent variable were used: decrease, no change, and increase in percent superficial cells. Adjusting for other variables was done as described above.

In further analyses to explore a possible dose response, treatment as a term in the models was replaced with each of three different measures of urinary soy estrogens: equal concentrations alone, an unweighted sum, or a weighted sum of concentrations with weights of 4 for daidzein, 8 for genistein, and 100 for equol, based on laboratory data on their relative estrogenicity (Shutt, D.A., and Cox, R.L., *J. Endocrinol.* 52:299 (1972); Tang, B. Y., and Adams, N. R., *J. Endocrinol.* 85:291 (1980)).

To evaluate possible interaction effects, i.e., that subsets of the population responded differently to the dietary intervention, terms were added that represented interactions with treatment for age, time since menopause, smoking status, weight, Quetelet's index, and estradiol level at baseline, to the models for FSH, LH, SHBG, and maturation index.

Of the ninety-seven women who began the study, three were found to be ineligible (one was still premenopausal, one was taking corticosteroids, and one was taking medication for diabetes). Three others dropped out during the study (two due to emergencies in their families, and one because she could not tolerate the soy foods). The remaining 91 women (66 in the soy diet group and 25 in control group) completed the intake and followup questionnaires, attended all clinic appointments, and collected 24-hour urine samples beginning the morning of their clinic appointment. More than 98% of the requested first-morning urine samples were successfully collected.

Compliance with the diet appeared to be good. Most (73%) reported eating all of their assigned foods. Eighteen women reported having eaten only part of their soy foods on at least one day, but only four women missed days completely (3 missed one day, 1 missed two days), and these occurred at the time of an illness. Consistent with these reports, urinary soy estrogens increased markedly for most women in the soy diet group (average of a 105-fold increase in the unweighted sum), but very little for the controls (average of a 2-fold increase which was not statistically significant). As expected there was little overlap between the control and soy diet groups, but the variation among the women in the soy diet group was broad, with some women showing extremely large increases, others showing more modest changes, and a few showing very little change. Despite efforts to recruit vegetarians, only four participated in the study. The two who were assigned to the soy diet group had low baseline levels and showed large increases in urinary isoflavone concentrations during the diet period.

As instructed, women maintained fairly stable body weights through the diet intervention period. The average weight change was a gain of 0.5 lbs. (0.4 for the diet intervention group and 0.9 for the controls). No one gained or lost more than five pounds and most (82%) varied by no more than two pounds from their baseline weight. Weight change was considered a potential covariate in all analyses. Endogenous estrogen levels, as reflected by serum estradiol concentrations, went down slightly during the study for both the diet-intervention and control groups. The decline was slightly larger for the diet-intervention group than for the controls (1.4 pg/ml vs. 0.9 pg/ml), but not significantly so. Change in estradiol level was considered a potential covariate in all analyses.

Changes over the four-week diet period in serum LH, FSH, SHBG, and in maturation index were determined. Adjustment for season, clinic, age, change in body weight, or change in estradiol concentration had little effect on these relationships. LH and FSH were predicted to decrease with soy intervention, and both did tend to decrease slightly (FSH more so than LH). However, controls also showed small average decreases, and the soy diet and control groups did not differ from each other ( $p=0.33$  for FSH;  $p=0.89$  for LH). SHBG was predicted to increase with soy intervention, but instead, SHBG tended to decrease for both the soy diet and control groups to a similar degree ( $p=0.89$ ). Maturation index was predicted to increase with soy intervention. Though there was a slight average increase in the soy diet group compared to a slight decline in the controls, the groups did not differ significantly ( $p=0.40$ ).

When vaginal epithelium data was examined further by focussing on superficial cells (the cells most indicative of estrogen stimulation) the changes were again in the predicted direction (increased superficial cells with dietary intervention). In unadjusted data 43 (68%) women in the soy diet group showed no change in percent superficial cells during the diet, 8 (13%) showed a decrease, and 12 (19%) showed an increase compared with 17 (71%), 5 (21%), and 2 (8%) in the respective categories of the controls ( $\chi^2_{df=2}=2.03$ ;  $p=0.36$ ). After adjustment for other factors with ordinal logistic regression, the same trend was determined: in comparison to controls women in the soy diet group tended to have no change or to have their proportion of superficial cells increase during the diet period rather than decrease (Odds Ratio=2.5 (0.8,7.8)), but the difference was consistent with chance ( $p<0.06$ ).

Urinary levels of the soy estrogens may provide a measure of biologically effective dose for women in the soy diet group. Therefore, evidence for a dose-response relationship between concentrations of urinary soy estrogens and estrogenic responses was evaluated. Concentration of urinary soy estrogens was not a better predictor of the outcome measures than treatment alone, whether the change in soy estrogens was modelled as a linear term or as four separate categories corresponding to quartiles of change. Adjustment for other variables did not affect this result.

Potential interactions were also considered to determine whether subsets of the study participants may have responded to the diet as predicted even though the soy diet group as a whole showed no clear estrogenic responses. Effects that might depend on age, time since menopause, smoking status, weight, Quetelet's index, and estradiol level at baseline were considered. Of the 24 tests conducted, two showed significant interaction effects ( $p<0.05$ ). One suggested that the diet/control comparison for change in FSH varied by age. Both soy diet and control groups tended to have small decreases in serum FSH concentrations during the diet period. However, the magnitude of decrease was positively correlated with age in the control group, while there was little variation with age in the diet intervention group. The second interaction suggested that the diet/control comparison for change in SHBG varied by smoking status. Whereas nonsmokers in both soy diet and control groups showed similar small decreases in SHBG, the diet smokers ( $n=10$ ) and control smokers ( $n=5$ ) differed from each other: the smokers in the control group had a small increase in SHBG, and the smokers in the soy diet group had a small decrease. Considering the multiple tests conducted, and that there was no biological basis for predicting such effects, these statistically significant interactions may have resulted from chance.

This Example relates to experiments designed to evaluate the beneficial effects of a combined estrogen/phytoestrogen dietary regimen on rats. More specifically, serum lipid profiles, as well as effects on the uterus and bone will be monitored. The architectural and hormonal milieu of the uterus has not been replicated successfully in any alternative to the *in vivo* model, because the endocrine and paracrine regulators of normal uterus are not completely understood. The small size, short lifespan, and ease of handling make rats ideal for scientific studies. The rat is a well-characterized model species for human diseases and has been used specifically to model uterine cancer. Endometrial hyperplasia can be induced in rats using the doses of Premarin described below. Rats are also well-characterized with respect to serum lipids and lipoproteins and are adequate for assessment of bone density.

Animals will be randomized by body weight into 12 dietary treatment groups of 10 animals each. Nine groups will undergo bilateral ovariectomy (OVX) by 45 days of age, whereas three groups will undergo sham ovariectomy.

TABLE 2

Group	Diet
1. Low Phytoestrogen + Sham OVX	Low-isoflavone soybean-based diet (equivalent on a caloric basis to 12.05 mg Genistein/woman/day), intact female
2. Low Phytoestrogen + OVX	Low-isoflavone soybean-based diet equivalent to the above diet, ovariectomized female
3. Low Phytoestrogen + OVX + Low Premarin	Low-isoflavone soybean-based diet + Premarin equivalent on a caloric basis to the above diet with the addition of 0.3125 mg Premarin/woman/day
4. Low Phytoestrogen + OVX + High Premarin	Low-isoflavone soybean-based diet + Premarin equivalent on a caloric basis to the above diet with the addition of 0.625 mg Premarin/woman/day
5. High Phytoestrogen + Sham OVX	High-isoflavone soybean-based diet (equivalent on a caloric basis to 117.06 mg Genistein/woman/day), intact female
6. High Phytoestrogen + OVX	High-isoflavone soybean-based diet, ovariectomized female
7. High Phytoestrogen + OVX + Low Premarin	High-isoflavone soybean-based diet + Premarin equivalent on a caloric basis to the above diet with the addition of 0.3125 mg Premarin/woman/day
8. High Phytoestrogen + OVX + High Premarin	High-isoflavone soybean-based diet + 0.625 mg Premarin/woman/day
9. Casein + Sham OVX	Casein-based diet, intact female, no Premarin
10. Casein + OVX	Casein-based diet, ovariectomized female, no Premarin
11. Casein + OVX + Low Premarin	Casein-based diet, ovariectomized female, + 0.3125 mg Premarin/woman/day
12. Casein + OVX + High Premarin	Casein-based diet, ovariectomized female, +0.625 mg Premarin/woman/day

In Table 2, doses of hormonal drugs have been computed from clinically used human doses as: human dose/1800 calories/woman/day—dose per calorie of diet. Doses arrived at by this means are, therefore, consistently scaled and are adjusted for metabolic rate. They are similar to the dose which would be arrived at by scaling on the basis of body surface area. The human equivalent isoflavone intake has

been estimated by the same equation. Purified diets will be formulated by the Comparative Medicine Clinical Research Center Diet Laboratory. Sufficient isoflavone-rich and isoflavone-poor soy protein is on hand to formulate all diets needed for this study.

Using rats treated as described above, the following hypotheses will be evaluated:

1. Premarin with phytoestrogen will result in lower levels of endometrial hyperplasia in rats relative to Premarin with casein.
2. Premarin with phytoestrogen will retain the bone loss prevention effects of Premarin alone.
3. Casein alone will result in loss of bone mass relative to Premarin and phytoestrogen treated groups.
4. Phytoestrogen diet alone will not cause endometrial hyperplasia relative to Premarin-treated and casein-fed controls.

#### Example 4

The studies described in this example are intended to examine the separate and combined effects of a traditional estrogen (estradiol) and a soybean phytoestrogen on coronary artery function and reproductive organ pathology in the cynomolgus monkey. Fifty-six adult female cynomolgus monkeys will be selected from the NIDDK breeding colony. All monkeys are consuming a moderately atherogenic diet (0.2 mg cholesterol/Cal of diet) and will continue to consume diets containing the same amount of cholesterol throughout the study.

All monkeys will be ovariectomized, then randomized to 5 treatment groups (n=14 each). Group 1 will receive atherogenic diet with no estrogen replacement and casein will be provided as the source of protein. Group 2 will receive atherogenic diet with 17 beta-estradiol added to the diet at an equivalent dose of 1 mg/day and casein will be provided as the source of protein. Group 3 will receive atherogenic diet, soybean protein (with plant estrogen) as source of protein (equivalent plant dose of 140 mg/day). Group 4 will receive atherogenic diet, estradiol and soybean as the source of protein in the diet. Treatment will be for a period of 5 months.

Prior to necropsy, a number of determinations will be made. Plasma lipid and lipoproteins (i.e., TPC, HDL-C and TG) will be measured once prior to treatment and then 2, 4 and 5 months after treatment. LDL molecular weight will be determined once, 5 months after treatment. Sex hormones (i.e., plasma estradiol, LH and FSH) will be determined once prior to treatment and then 2 months and 5 months after treatment. Plasma gonadotropin concentrations will be measured once, 5 months after treatment. Vaginal Cytology will be done 2 and 5 months after treatment. To determine body fat, ponderosity measurements will be done before and 2 and 5 months after treatment. Serum will be collected and stored for Lp(a) determination.

Monkeys will be anesthetized 2 months after starting treatment and the left iliac artery denuded using a balloon catheter. The procedure will be done aseptically using standardized techniques for "balloon injury".

Immediately prior to necropsy, monkeys will be anesthetized and a catheter advanced from the right femoral artery to the left main coronary artery for measurements of coronary artery diameter using angiographic techniques. Vascular responses of large epicardial coronary arteries will be measured in response to intracoronary infusion of endothe-

lium-dependent and independent agonists. Simultaneous measurements of coronary artery velocity will be done using Doppler techniques to evaluate the effects of treatment on coronary blood flow and reactivity of the coronary microvasculature.

Immediately following the coronary reactivity studies, standard sections of breast tissue, uterus, cervix and vagina will be removed for processing as described below. Routine histology (cell size and character, myometrial thickness and glandular development), estrogen receptor expression, progesterone receptor expression, and Ki-67/MIB proliferation marker studies will be done on the tissue. The monkey will then be prepared for whole body perfusion-fixation at physiologic pressure with 10% neutral buffered formalin. The heart will be removed and coronary arteries prepared for histomorphometric analysis. The two common iliac arteries will also be processed for histomorphometric analysis of neointimal formation after balloon injury.

The tissue sections described in the preceding paragraph will be analyzed for: 1) the development of hyperplastic, dysplastic, or neoplastic lesions in breast and endometrium; 2) effects of treatments on estrogen and progesterone receptor status of mammary epithelium and endometrium; and 3) effects of treatment on expression of an endogenous marker of cellular proliferation in mammary and endometrial cells. Hyperplasia and dysplasia will be assessed subjectively and by computer-assisted image analysis of glandular morphology and nuclear size and shape. Estrogen and progesterone receptor status and a marker of cellular proliferation (Ki-67/MIB) will be assessed by immunohistochemistry followed by stereologic quantification of proportions of labeled cells. Parallel pathologic and immunolabeling studies of endometrial responses will be done, allowing a comparative assessment of the two tissues in individual animals.

We claim:

1. A pharmaceutical composition for oral delivery comprising a combination of mammalian estrogen and soy-derived phytoestrogen in an amount sufficient to reduce the risk of coronary heart disease and osteoporosis in women.
2. The pharmaceutical composition of claim 1, wherein the amount sufficient to reduce the risk of coronary heart disease and osteoporosis in women is about 1-2 mg of mammalian estrogen combined with about 25-100 mg phytoestrogen.
3. A pharmaceutical composition of claim 1 which is in pill or capsule form.
4. A pharmaceutical composition of claim 1 wherein the soy-derived mammalian estrogen is estradiol.
5. A pharmaceutical composition of claim 1 wherein the phytoestrogen is an isoflavone.
6. A pharmaceutical composition of claim 5 wherein the isoflavone is genistein.
7. A pharmaceutical composition of claim 5 wherein the isoflavone is daidzein.
8. A method for reducing the risk of coronary heart disease and osteoporosis in women, the method comprising administering orally a combination of mammalian estrogen and soy-derived phytoestrogen in a therapeutically effective amount.
9. The method of claim 8 wherein the therapeutically effective amount is a daily dosage of mammalian estrogen of about 1-2 mg, and soy-derived phytoestrogen of about 25-100 mg.
10. A method of claim 8 wherein the mammalian estrogen is estradiol.
11. A method of claim 8 wherein the soy-derived phytoestrogen is an isoflavone.

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12. A method of claim 11 wherein the isoflavone is genistein.

13. A method of claim 11 wherein the isoflavone is daidzein.

14. An estrogen replacement therapy regimen, comprising the oral coadministration of a combination of mammalian estrogen and soy-derived phytoestrogen in a therapeutically effective amount.

15. The estrogen replacement therapy regimen of claim 14 wherein the therapeutically effective amount is a daily dosage of mammalian estrogen of about 1–2 mg, and soy-derived phytoestrogen of about 25–100 mg.

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16. A method of claim 14 wherein the mammalian estrogen is estradiol.

17. A method of claim 14 wherein the soy-derived phytoestrogen is an isoflavone.

18. A method of claim 17 wherein the isoflavone is genistein.

19. A method of claim 17 wherein the isoflavone is daidzein.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 5,516,528

DATED : May 14, 1996

INVENTOR(S) : Claude L. Hughes, Edna C. Henley and  
Thomas B. Clarkson

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 14:

In Claim 48, delete "soy-derived".

In Claim 49, after the word "the", insert ---soy-derived---

Signed and Sealed this  
Fifth Day of November, 1996

Attest:



BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks



US005376652A

## United States Patent [19]

Javitt

[11] Patent Number: 5,376,652

[45] Date of Patent: Dec. 27, 1994

- [54] ADMINISTRATION OF A  
27-HYDROXYCHOLESTEROL OR RELATED  
COMPOUND OR  
STEROL-27-HYDROXYLASE STIMULANT  
TO PREVENT RESTENOSIS FOLLOWING  
VASCULAR ENDOTHELIAL INJURY

[75] Inventor: Norman B. Javitt, New York, N.Y.

[73] Assignee: New York University Medical Center,  
New York, N.Y.

[21] Appl. No.: 159,226

[22] Filed: Nov. 30, 1993

[51] Int. Cl.<sup>5</sup> ..... A61K 31/575

[52] U.S. Cl. .... 514/177; 514/178;  
514/181; 514/182

[58] Field of Search ..... 514/182, 177, 178, 181,  
514/58

- [56] References Cited

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Macpeak & Seas

[57]

## ABSTRACT

A method for preventing or reducing restenosis  
wherein a 27-hydroxycholesterol or a 25,26 and/or  
27-aminocholesterol, or a sterol 27-hydroxylase stimu-  
lant is administered in a restenosis preventing and/or  
reducing amount.

24 Claims, No Drawings

**ADMINISTRATION OF A  
27-HYDROXYCHOLESTEROL OR RELATED  
COMPOUND OR STEROL-27-HYDROXYLASE  
STIMULANT TO PREVENT RESTENOSIS  
FOLLOWING VASCULAR ENDOTHELIAL  
INJURY**

**BACKGROUND OF THE INVENTION**

Various surgical bypass and angiographic procedures are routinely employed for increasing blood flow to an organ, usually the heart. These operative and non-operative procedures injure to a greater or lesser extent the interior wall of the lumen of the blood vessel at the target site. This endothelial injury often leads through a cascade of events to restenosis. For example, balloon, laser or rotameter angioplasty, in which a catheter is inserted into the arterial system to place a balloon, laser or blade at the stenosis, is quite successful in widening a narrowed area of a blood vessel lumen. However, endothelial injury occurs at the site of the lesion, leading to restenosis in an estimated 20-40% of patients.

It has been shown in animals that endothelial injury initiates a process that leads to narrowing of the injured artery (stenosis), and this model is related and is used to study the events that occur following endothelial injury.

The major current theory for explaining restenosis is that once the endothelial cells are injured or removed by the invasive procedure, circulating platelets cover the denuded areas and release potent growth factors, such as platelet derived growth factor, which stimulate the growth and migration of underlying smooth muscle cells. Other growth factors such as fibroblast growth factors have also been implicated. For these reasons, anti-growth factors are being evaluated for the prevention of atherosclerosis.

Many factors are thought to potentially participate in restenosis. Further, hemodynamic forces responsible for the original lesion are not generally alleviated by angioplasty and may be aggravated at plaque disruption. The thrombo-resistant nature of the arterial lumen is reduced due to the generation of markedly thrombogenic surfaces of complex geometrical configuration, and changed permeability characteristics permitting possible direct interaction between blood-borne elements such as the aforementioned platelets and the arterial lumen. In summary, the surgical and angiographic procedures necessarily result in injury to vessel walls, which results in restenosis in 20-40% of patients.

27-hydroxycholesterol (cholest-5-ene-3 $\beta$ ,27-diol) is normally present in biological fluid after neonatal life. Recently, the IUB changed certain rules of nomenclature, and the compound now referred to as 27-hydroxycholesterol was previously called 26-hydroxycholesterol. Two methyl groups are attached to carbon number 25 of cholesterol, but only one can be enzymatically hydroxylated, which was previously named carbon number 26, but is now named as carbon number 27.

U.S. Pat. No. 4,427,688 by Javitt describes the administration of 26-hydroxycholesterol (sic., 27-hydroxycholesterol) and various derivatives and analogs thereof for reducing cholesterol synthesis and/or cholesterol accumulation in the body tissues; hence, teaching the use of 27-hydroxycholesterol compounds for the treatment of atherosclerosis. Thereafter, as disclosed in U.S. Pat. No. 4,939,134, Javitt, et al. discovered that 27-aminocholesterol and certain amino-substituted analogs

and derivatives thereof, are more potent inhibitors of cholesterol synthesis and accumulation than 27-hydroxycholesterol.

Javitt filed Japanese application 107488/82 in 1982, published as 019206/91 on Nov. 14, 1991 ("JPA"), largely corresponding in disclosure to U.S. Pat. No. 4,427,688 with insertion of additional information for further supporting use of 27-hydroxycholesterol in treatment of atherosclerosis. The JPA notes that Kandutsch, et al., *Science*, 201, 498 (1978) mentioned that oxygenated cholesterol has an inhibitory effect on the proliferation of fibroblasts and lymphocytes in vitro, perhaps by inhibiting HMG CO-A reductase, the rate-limiting enzyme in cholesterol biosynthesis, which is consistent with the idea that cholesterol is essential to cell proliferation. Javitt tested this theory by seeding hamster aortic smooth muscle cells at low density in culture wells and Coulter counting control and 27-hydroxycholesterol exposed cells six days later. The 27-hydroxycholesterol at the tested concentration inhibited the proliferation by about 50%. Although a potential lead, in vitro muscle cell proliferation inhibition, in itself, does not teach nor suggest the use of the same substance for preventing restenosis in vivo. Indeed, some have interpreted the inhibitory effect by oxysterols on vascular smooth muscle cells as a toxic effect. Zhou, et al., *Proc. Soc. Exp. Biol. Med.*, 202:75-80; Nassen, et al., *Biochem. Internat.*, 14:71-84. Also see Baranowski, et al., *Atherosclerosis*, 41:255-260. Further, it has been recently reported that high doses of Lovastatin, a potent cholesterol lowering drug, in a randomized, double blind placebo controlled trial, did not decrease restenosis six months following percutaneous transluminal coronary angioplasty, although the Lovastatin did markedly decrease LDL-cholesterol level, as expected. Thus, as of today, the cholesterol lowering biological activity of a drug, while perhaps indicating potential use in the treatment of atherosclerosis, is not predictive nor suggestive of use for combating restenosis. As discussed above, restenosis is a multifaceted phenomena, distinct from atherosclerosis, and the mechanism of which is at best only partially understood.

**SUMMARY OF THE INVENTION**

Hereinafter, the currently accepted nomenclature for the sterol nucleus involved herein, 27-hydroxycholesterol, is employed. It is understood that this compound is identical to the compound named 26-hydroxycholesterol in the prior art discussed above as well as other prior art.

It has now been found that 27-hydroxycholesterol effectively reduces restenosis following injury to the blood vessel lumen which occurs when the lumen is widened by catheter procedure. Therefore, it is an object of the present invention to provide a method for reducing the instance of restenosis which occurs following surgical by-pass procedures and percutaneous angiographic procedures. Further, it is expected that related compounds such as 25-, 26-, and/or 27-aminocholesterol will have a similar effect on the blood vessel lumen.

Another object of the present invention is to provide a method for reducing the occurrence of restenosis after balloon, laser or rotameter angioplasty. A further aspect of the present invention involves the administration of a 27-hydroxycholesterol or related compound immedi-

ately following injury to the lumen of a blood vessel as a result of a mechanically widening thereof, and to continue to administer said compound to a patient in a maintenance dosage to prevent restenosis.

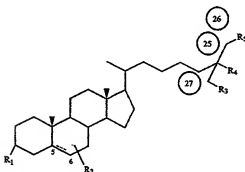
In still another embodiment of this invention, the 27-hydroxycholesterol or related compound is administered orally or intravenously, preferably intravenously dissolved in an aqueous solution of a  $\beta$ -cyclodextrin such as 2-hydroxypropyl- $\beta$ -cyclodextrin.

In another embodiment of the present invention, a sterol 27-hydroxylase stimulant is administered to thereby increase the synthesis of 27-hydroxycholesterol in the vascular tissue, this aspect of the invention based on a finding of sterol 27-hydroxylase activity in aortic endothelial cells.

Other objects of the invention will be apparent to the skilled artisan from the detailed description of the invention hereinbelow.

#### DETAILED DESCRIPTION OF THE INVENTION

The compounds for use in the practice of the present invention are, in general, known in the art, and may be represented by the following formula:



wherein  $R_1$  is hydroxyl or keto;  $R_2$  is hydrogen, hydroxyl or keto;  $R_3$  is hydroxyl, hydrogen or amino;  $R_4$  and  $R_5$  are hydrogen or amino; with the proviso that when  $R_3$  is not hydroxyl, at least one of  $R_3$ ,  $R_4$  and  $R_5$  is amino ( $-NH_2$ ) and the other(s) of them are hydrogen or amino, and pharmaceutically acceptable derivatives and salts thereof. In formula (I),  $R_3$  is substituted at position 27.

A preferred group of compounds for use in the practice of the present invention are those within formula (I) wherein the 27-position ( $R_3$ ) is substituted by hydroxyl or amino and each of  $R_4$  and  $R_5$  is hydrogen.

At this time, the most preferred compound for use in the inventive process for reducing and/or preventing restenosis is 27-hydroxycholesterol.

Other compounds usable herein include 25-aminocholesterol, 26-aminocholesterol, 27-aminocholesterol, 27-nor-25-amino-cholesterol, 25-amino-cholesta-4,6-dien-3-one, 25-amino-cholesta-4-en-3-one, 22-aminocholesta-5-en-3-one, 20-amino-25,26,27-trinorcholesta-5-en-3-one, 25-amino-cholesta-3,5-dien-7-one.

For administration to a patient, the compounds of the present invention can be provided, per se, or as the mono and diesterified derivatives and other pharmaceutically acceptable derivatives thereof such as the mono-

and diesters. Most usually, fatty acid, the same or analogous to those naturally occurring, would be used to form the esters, but other inorganic and organic esters, such as acetates, the sulfates, carbonates and glucuronides, routinely employed in preparing pharmaceutically acceptable esters could be used. Esterification and/or etherification can occur at the 3- and/or 27-position, or at carbon positions 6 or 7 when  $R_2$  is hydroxyl. Aryl and/or alkyl ethers, such as methyl, ethyl or cycloalkyl (i.e., cyclopentyl ethers) are contemplated. Furthermore, acid salts and various substituted compounds, for example, those containing elements such as fluorine commonly used in modification of steroid-type compounds, as long as pharmaceutically acceptable, can be used.

Administration can be through the use of liquid and solid formulations and also through the use of injectables, such as intravenous injectables, wherein conventional pharmaceutical carriers would be employed.

Suitable pharmaceutical preparations include tablets, capsules, oral liquids and parenteral injectables. Tablet and capsule formulations can be employed utilizing conventional diluents, excipients and the like such as lactose in conventional capsule and tablet-making procedures. Parenteral injections could employ solvents conventionally used with lipid-soluble materials, or a salt of the sterol could be prepared, at least some of which should be soluble in aqueous solutions.

It has been difficult to form aqueous solutions of the compounds found herein for parenteral administration. None of the vehicles commonly used to solubilize steroids and bile acids, such as propylene glycol, ethanol, dimethyl sulfoxide or dimethyl formamide, is able to maintain solubility when diluted with aqueous media. As disclosed by DeCaprio, Yen and Jevitt, *Journal of Lipid Research*, Vol. 33, pp. 441-443, 1992, 27-hydroxycholesterol and it is expected the related compounds involved herein, can be stabilized in aqueous media by inclusion of a cyclodextrin therein. It has been theorized that the cyclic structure of the cyclodextrin provides a lipophilic interior in which compounds that have limited aqueous solubility will form a soluble complex. The  $\beta$ -cyclodextrins usable for this purpose are known in the art and are inclusive of the 2-hydroxypropyl- $\beta$ -cyclodextrin described by DeCaprio, et al., supra. Other non-toxic cyclodextrins would be usable.

The compounds of the present invention are administered in amounts ranging from 10 mg/kg to 100 mg/kg, preferably about 20 mg/kg to 40 mg/kg, 1 to 3 times a day.

In one embodiment of the present invention, the compound of the present invention is administered as a bolus, employing a dosage toward the upper end of the above dosage range, immediately prior to, during and/or following the blood vessel lumen widening, followed by reduction to a maintenance dosage toward the lower end of the above dosage range. It is contemplated that the maintenance dosage would continue over a prolonged period of time of, for example, 1 to 5 months.

The following example is provided to illustrate the above-described aspect of the present invention:

#### EXAMPLE 1

##### Vascular Injury Model

New Zealand white rabbits weighing 2.6 to 5.1 kg were anesthetized with intramuscular ketamine 35 mg/kg. Additional injections of ketamine (100 mg/cc)

and xylazine (20 mg/cc) in a 50/50 mixture were given as necessary in 1 ml increments. Keflin (Eli Lilly & co.), 30/mg/kg was given intravenously (IV). A longitudinal incision was made on the medial aspect of the distal hind limb to expose the greater saphenous artery. Arteriotomy was performed, and a 3-F Fogarty embolotomy catheter (Edwards laboratories, Santa Ana, Calif.) was introduced and advanced to the level of the diaphragmatic abdominal aorta. The catheter was withdrawn from the abdominal aorta with the balloon inflated to a pressure of about 20 mmHg. This maneuver was repeated for a total of three passes. The catheter was removed, and the saphenous artery was ligated. The wound was irrigated and closed with 4-0 Dexon suture.

#### Specimen Analysis

The abdominal aortas were fixed by perfusion with glutaraldehyde at physiological pressure via a catheter (14G Intra cath) placed in the left ventricle. One micron longitudinal sections of epoxy embedded aorta specimens were cut, stained, and computer imaged. The entire intimal and medial areas in more than 2 sections per specimen were measured using Lucia computer calculation (Micro Brightfield, Inc.). The degree of intimal thickening was determined by calculating the intimal to medial area (I/M) ratio. Statistical significance of the difference in intimal/medial ratio between groups was calculated using student t-test.

#### Run I

Using the above procedure, a study was carried out employing five control balloon injury rabbits and two groups, each of five balloon injury test rabbits, for receiving 27-hydroxycholesterol or suramin, the latter having been shown to inhibit intimal proliferation.

On the day before surgery, 10 mg 27-hydroxycholesterol dissolved in 1.0 ml of 45% aqueous solution of 2-hydroxypropyl- $\beta$ -cyclodextrin (HPBCD) was administered intravenously to one group of five test rabbits. Suramin was administered to the other test group. bFGF was administered throughout the testing period.

On the day of surgery, a short time prior to the balloon angioplasty, the five test rabbits were administered another 5 mg of 27-hydroxycholesterol in 0.5 ml HPBCD, and the same dosage was administered to each of the five test rabbits twice a day on days 1 to 14 following the day of balloon angioplasty, in the form of 5 mg 27-hydroxycholesterol in 0.5 ml 45% HPBCD twice a day. On day 14, the above specimen analysis was carried out on the fifteen rabbits, with the following results.

	QUANTITATIVE HISTOLOGICAL EVALUATION OF ARTERIAL WALL 14 DAYS		
	Mean Intima/Media Ratio <sup>1</sup>		
	5-DAY	14-DAY	
Control (28 rabbits)	0.094 $\pm$ 0.006	0.5562 $\pm$ 0.0204	
Suramin Administration		0.4089 $\pm$ 0.0164 <sup>2</sup>	
27-Hydroxycholesterol Administration		0.4872 $\pm$ 0.0238	

<sup>1</sup>Ratio of thickness of intima and media of artery wall  
<sup>2</sup>p < 0.05

This run suggests an improved intima/media ratio through the administration of 27-hydroxycholesterol. However, the results were not as good as with suramin and when calculated, the difference between the con-

trol group and the 27-hydroxycholesterol group was not statistically significant. With a suggestion of utility, a further run was carried out with increased

#### Run II 27-hydroxycholesterol dosage.

Control (vehicle alone) and 27-hydroxycholesterol test rabbits were used as in Run I, with the exception that each test rabbit received 100 mg 27-hydroxycholesterol dissolved in 5.0 ml 45% HPBCD subcutaneously on the day before surgery, the day of surgery and on days 1 to 14 following surgery.

	QUANTITATIVE HISTOLOGICAL EVALUATION OF ARTERIAL WALL 14 DAYS	
	Mean Intima/Media Ratio	
	Control (28 rabbits)	Test (20 rabbits)
	0.5209 $\pm$ 0.0171	0.2880 $\pm$ 0.0471

The 27-hydroxycholesterol reduced degree of intimal thickening by nearly  $\frac{1}{2}$  as compared with the control group.

In a second aspect of the present invention, a sterol-27-hydroxylase stimulant is administered to increase the sterol-27-hydroxylase activity present in vascular tissue, the presence thereof in vascular tissue being heretofore unknown. In this manner not only is the available amount of 27-hydroxycholesterol enhanced since it is one of the major metabolites from sterol-27-hydroxylase activity, but the 27-hydroxycholesterol is produced in the cells at the location where it is best utilized by the body in reducing and/or preventing restenosis.

The sterol-27-hydroxylase activity in bovine aortic endothelial (BAE) cells in culture has been compared with that in Hep G2 cells and in chinese hamster ovary (CHO) cells using identical culture conditions. The total enzyme activity of BAE cells (3.0 nmol/72 h/mg cell protein) was comparable with that of Hep G2 cells (4.0 nmol/72 h/mg protein) and both values were significantly greater than that in CHO cells (0.002 nmol/72 h/mg protein). The enzyme was identified in BAE cells by Western blotting using an antibody of proven specificity, and its metabolites 27-hydroxycholesterol and 3 $\beta$ -hydroxy-5-cholestenic acid were identified by mass spectrum analysis. The presence of the enzyme in endothelium provides a mechanism for providing the biologic effects of 27-hydroxycholesterol in vascular tissue.

#### EXAMPLE 2

##### Cell Culture

Bovine aortic endothelial (BAE) cells obtained from a slaughterhouse were plated at low density ( $2 \times 10^5$  cells/cm<sup>2</sup>) in 100-mm dishes and were grown to confluence in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin, and 50 units/ml streptomycin at 37°C in a 5% CO<sub>2</sub> atmosphere. The confluent monolayer was rinsed once with Hank's balanced salt solution, and 4 ml of DMEM containing 10% delipidated FBS and either 20  $\mu$ M cholesterol dissolved in 2-hydroxypropyl- $\beta$ -cyclodextrin or an equivalent amount of vehicle alone was added to each dish. Hep G2 cells and chinese hamster ovary cells (CHO) were cultured under identical conditions and for the same length of time. At 24-h intervals the media and cells were harvested. The me-

dium obtained from each dish was analyzed for metabolites. Cells were pooled for immunoblot analysis.

#### Western Blotting

Samples were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and were transferred onto a nitrocellulose membrane by an electrophoretic technique. Antibody was raised in rabbits against residues 15 to 28 of the 27-hydroxylase protein. Visualization was accomplished using an alkaline phosphatase-conjugated goat and anti-rabbit antibody followed by the Rad-free kit for colorimetric detection of Western blots (Schleicher & Schuell, Keene, N.H.).

Several attempts were made to detect the 56-kb 27-hydroxylase protein in mitochondria prepared from BAE cells, but despite the use of protease inhibitors the predominant immunoreactive band was detected at 35 kd, with occasionally a faint band at 56 kd. Boiling freshly harvested whole cells in loading buffer appears to have prevented proteolysis.

#### GLC-MS Analysis

##### Sample Preparation

To 1 ml of harvested medium, internal standards (500 ng each) of deuterated 27-hydroxycholesterol, 3 $\beta$ -hydroxy-5-cholestenic acid (prepared by Jones oxidation of the 3-monoacetate of the deuterated 27-hydroxy-sterol), and <sup>13</sup>C-3 $\beta$ -hydroxy-5-cholestenic acid were added and allowed to equilibrate for 30 min at room temperature. Following acidification and extraction into ethyl acetate, the dried residue was saponified. In some studies solvolysis was also done prior to extraction. The dried extract was applied to a Silica gel G TLC plate together with authentic standards in parallel lanes; after development (chloroform/acetone, 97:3) the standards were visualized by spraying with phosphomolybdic acid and the appropriate areas of the plate were removed for elution of 27-hydroxycholesterol and the C<sub>27</sub> and C<sub>24</sub> acids. The diacetate of 27-hydroxycholesterol and methyl acetates of the C<sub>27</sub> and C<sub>24</sub> acids were then prepared using dimethoxypropane/HCl for methylation and pyridine/acetic anhydride for acetylation. It was found that complete methylation of the C<sub>27</sub> acid with dimethoxypropane/HCl took longer than that of the C<sub>24</sub> acid. Therefore methylation was allowed to proceed at room temperature overnight (approximately 18 h). Formation of a 3-methoxy derivative by this prolonged methylation procedure was not detected.

##### Isotope Ratio Mass Spectrometry

Using a Hewlett-Packard GLC-MS (Model #5890-5970) and a fused silica column (CP-sil 19 CB, 0.25 mm i.d., 25 m length; Chrompack, Karis, N.J.), the appropriate TLC fractions were injected in the splitless mode with temperature programming from 260° C. to 270° C. at 1.0° C./min and a column head pressure of 5 psi.

To quantify 27-hydroxycholesterol the detector was programmed in the simultaneous ion monitoring mode for m/z 426 [mol ion diacetate = 486-60 (acetate)] and m/z 430, and the amount of endogenous 27-hydroxycholesterol was calculated from the respective areas. For the C<sub>27</sub> acid the ion pair that was used was m/z 412 [methyl ester acetate mol ion = 476-60 (acetate)] and m/z 416, and for the C<sub>24</sub> acid m/z 370 [mol ion methyl ester acetate = 430-60 (acetate)] and m/z 373.

#### Results

Both the spectra and the retention times of 27-hydroxycholesterol and of 3 $\beta$ -hydroxy-5-cholestenic acid isolated from the sterol-free medium that was in contact with BAE cells for 72 h are identical to authentic standards of the diacetate and methyl ester diacetate derivatives, respectively.

After the identity of these compounds was established by complete spectrum analysis, an isotope ratio program was used to compare their rates of synthesis in sterol-free and cholesterol-supplemented medium. As shown in Table 1, medium containing 20 nmol/ml of cholesterol yielded a much greater amount of metabolites than sterol-free medium. At 72 h the metabolites represented approximately 5.8% of the cholesterol added to the medium  $[(1.029 + 0.211) - (0.103 + 0.079) \times 100 + 20]$ . Although the amount of 27-hydroxycholesterol in the medium was relatively constant from 24 to 72 h, a progressive increase in the amount of 3 $\beta$ -hydroxy-5-cholestenic acid occurred. For BAE cells maintained in nonsupplemented medium the proportion of C<sub>27</sub> acid rose from 12% at 24 h to 43% at 72 h. In contrast, although the absolute amount of the C<sub>27</sub> bile acid that was synthesized was greater in cholesterol-supplemented medium, it represented only 3.8% of total metabolite at 24 h and increased to 17% at 72 h.

The activity of sterol 27-hydroxylase in BAE cells was compared with that in Hep G2 and CHO cells using the cholesterol-supplemented medium. As shown in Table 2, the amount of 27-hydroxycholesterol in the medium collected from BAE cells at 72 h was greater than that from Hep G2 cells. The amount present in medium from CHO cells was below our limit of detection (10 ng per assay).

The medium from CHO cells always contained a small amount of 3 $\beta$ -hydroxy-5-cholestenic acid, which was much less than that found in the medium from Hep G2 or BAE cells.

Because Hep G2 cells synthesize 3 $\beta$ -hydroxy-5-cholestenic acid from 27-hydroxycholesterol, the medium from all the cell lines was analyzed for this derivative before and after solvolysis. No increase in the yield of 3 $\beta$ -hydroxy-5-cholestenic acid was obtained after solvolysis of media derived from Hep G2 or the other cell lines. Medium from Hep G2 cells was found to contain 3 $\beta$ -hydroxy-5-cholestenic acid, which increased in amount following solvolysis.

Since all the metabolic products are derived from the sterol 27-hydroxylase activity of the cells, the total amounts produced by Hep G2 and BAE cells are comparable and are much greater than that from CHO cells.

TABLE 1

Synthesis of 27-hydroxycholesterol and 3 $\beta$ -hydroxy-5-cholestenic acid by BAE Cells Time course and effect of cholesterol added to the medium			
Culture Medium	24 h	42 h	72 h
27-hydroxycholesterol (nmol/ml medium)			
Delipidated FBS <sup>a</sup>	89 $\pm$ 12 <sup>b</sup>	98 $\pm$ 11	103 $\pm$ 9
(n = 6) <sup>b</sup>			
+20 nmol/ml cholesterol	1089 $\pm$ 111	1064 $\pm$ 161	1029 $\pm$ 99
3 $\beta$ -hydroxy-5-cholestenic acid (nmol/ml medium)			
Delipidated FBS	11.9 $\pm$ 1.3	32.0 $\pm$ 1.9	78.6 $\pm$ 11
+20 nmol/ml cholesterol	42.5 $\pm$ 6.1	112 $\pm$ 12.4	211 $\pm$ 48.3

TABLE 1-continued

Synthesis of 27-hydroxycholesterol and 3 $\beta$ -hydroxy-5-cholestenic acid by BAE Cells: Time course and effect of cholesterol added to the medium

Culture Medium	24 h	48 h	72 h
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terol  
 Delipidated fetal bovine serum  
 Number of dishes  
 Mean  $\pm$  standard deviation

TABLE 2

Cells <sup>a</sup>	Comparison of Sterol 27-hydroxylase activity in BAE, Hep G2 and CHO cells		
	Metabolites Derived from Sterol 27-Hydroxylase (pmol/mg/cell protein)		
	27OH-cho <sup>b</sup>	3 $\beta$ OH-5-cholestenic a.	Total
BAE (n = 6) <sup>c</sup>	2555 $\pm$ 348	474 $\pm$ 118	not detected
Hep G2 (n = 6)	1622 $\pm$ 291	471 $\pm$ 126	1940 $\pm$ 270 <sup>d</sup>
CHO (n = 3)	not detected	2 $\pm$ 1	not detected

<sup>a</sup>All cells were maintained for 72 h in DMEM enriched with 10% delipidated FBS containing 20  $\mu$ M cholesterol.

<sup>b</sup>27OH-cho<sup>b</sup> = 27-hydroxycholesterol; 3 $\beta$ OH-cholestenic a. = 3 $\beta$ -hydroxy-5-cholestenic acid; 3 $\beta$ OH-cholestenic a. = 3 $\beta$ -hydroxy-5-cholestenic acid.

<sup>c</sup>Number of dishes.  
<sup>d</sup>Mean value of 2 dishes after solvolysis.

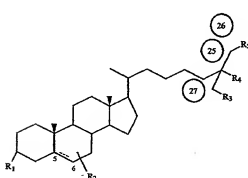
From the above, the positive effects of this invention on restenosis can be provided by stimulating the sterol 27-hydroxylase activity of the vascular endothelium. Various stimulatory mechanisms are known, such as by the administration of steroid hormones, such as the naturally-occurring sex hormones estrogen and testosterone.

The skilled artisan will be able to select other naturally-occurring and synthetic steroid hormones for use in providing a sterol 27-hydroxylase stimulant effect.

Variations of the invention will be apparent to the skilled artisan.

What is claimed is:

1. In a process wherein in a mammal, a therapeutic procedure is carried out to reduce or remove a stenosis present within a lumen of a blood vessel, the improvement to prevent restenosis which comprises administering to the mammal a restenosis preventing amount of a compound of formula (I)

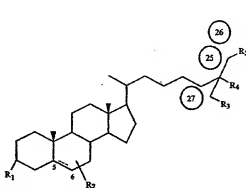


wherein R<sub>1</sub> is hydroxyl or keto; R<sub>2</sub> is hydrogen, hydroxyl or keto; R<sub>3</sub> is hydroxyl, hydrogen or amino; R<sub>4</sub> and R<sub>5</sub> are hydrogen or amino; with the proviso that when R<sub>3</sub> is hydroxyl both R<sub>4</sub> and R<sub>5</sub> are hydrogen and

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when R<sub>3</sub> is not hydroxyl, at least one of R<sub>4</sub>, R<sub>4</sub> and R<sub>5</sub> is amino (—NH<sub>2</sub>) and the other(s) of them are hydrogen or amino, and pharmaceutically acceptable derivatives and salts thereof.

2. In a process wherein in a mammal, a therapeutic procedure is carried out to reduce or remove a stenosis present within a lumen of a blood vessel, the improvement to reduce the degree of restenosis which comprises administering to the mammal a restenosis reducing amount of a compound of formula (I)



wherein R<sub>1</sub> is hydroxyl or keto; R<sub>2</sub> is hydrogen, hydroxyl or keto; R<sub>3</sub> is hydroxyl, hydrogen or amino; R<sub>4</sub> and R<sub>5</sub> are hydrogen or amino; with the proviso that when R<sub>3</sub> is hydroxyl both R<sub>4</sub> and R<sub>5</sub> are hydrogen and when R<sub>3</sub> is not hydroxyl, at least one of R<sub>4</sub>, R<sub>4</sub> and R<sub>5</sub> is amino (—NH<sub>2</sub>) and the other(s) of them are hydrogen or amino, and pharmaceutically acceptable derivatives and salts thereof.

3. The process of claim 1, wherein R<sub>3</sub> is hydroxyl or amino and each of R<sub>4</sub> and R<sub>5</sub> is hydrogen.

4. The process of claim 2, wherein R<sub>3</sub> is hydroxyl or amino and each of R<sub>4</sub> and R<sub>5</sub> is hydrogen.

5. The process of claim 1, wherein the compound of formula (I) administered is 27-hydroxycholesterol.

6. The process of claim 2, wherein the compound of formula (I) administered is 27-hydroxycholesterol.

7. The process of claim 1, wherein the compound of formula (I) is administered in a pharmaceutically acceptable carrier comprising an aqueous medium containing a pharmaceutically acceptable cyclodextrin in sufficient amount to stabilize the compound of formula (I) in the aqueous medium.

8. The process of claim 2, wherein the compound of formula (I) is administered in a pharmaceutically acceptable carrier comprising an aqueous medium containing a pharmaceutically acceptable cyclodextrin in sufficient amount to stabilize the compound of formula (I) in the aqueous medium.

9. The process of claim 1, wherein the therapeutic procedure is a surgical procedure.

10. The process of claim 2, wherein the therapeutic procedure is a surgical procedure.

11. The process of claim 1, wherein the therapeutic procedure is balloon, laser or rotameter angioplasty.

12. The process of claim 2, wherein the therapeutic procedure is balloon, laser or rotameter angioplasty.

13. The process of claim 1, wherein the therapeutic procedure is balloon angioplasty.

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14. The process of claim 2, wherein the therapeutic procedure is balloon angioplasty.

15. The process of claim 1, wherein the compound of claim 1 is administered in an amount of about 10 to 100 mg/kg 1 to 3 times a day.

16. The process of claim 2, wherein the compound of claim 1 is administered in an amount of about 10 to 100 mg/kg 1 to 3 times a day.

17. The process of claim 1, wherein the compound of formula (I) is administered as the compound itself or as a mono- or di-ester, or mono- or di-ether thereof.

18. The process of claim 2, wherein the compound of formula (I) is administered as the compound itself or as a mono- or di-ester, or mono- or di-ether thereof.

19. The process of claim 1 wherein the compound of formula (I) is administered prior to, during and/or after the therapeutic procedure.

20. The process of claim 2 wherein the compound of formula (I) is administered prior to, during and/or after the therapeutic procedure.

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21. The process of claim 1 wherein the compound of formula (I) is administered after the therapeutic procedure.

22. The process of claim 2 wherein the compound of formula (I) is administered after the therapeutic procedure.

23. In a process wherein in a mammal, a therapeutic procedure is carried out to reduce or remove a stenosis present within a lumen of a blood vessel, the improvement to reduce the degree of restenosis which comprises administering to the mammal a sterol 27-hydroxylase stimulant in an amount sufficient for reducing said degree of restenosis.

24. In a process wherein in a mammal, a therapeutic procedure is carried out to reduce or remove a stenosis present within a lumen of a blood vessel, the improvement to prevent restenosis which comprises administering to the mammal a sterol 27-hydroxylase stimulant in an amount sufficient for preventing restenosis.

**(x) Related proceedings appendix**

None.